A comparative study of age-dependent susceptibility to the transmissible spongiform encephalopathies

Suzanne Gail St.Rose

Thesis submitted for the degree of
Doctor of Philosophy

The University of Edinburgh
2006

Declaration

I hereby declare that the work presented in this thesis is my own except where otherwise stated. No part of this work has been, or will be, submitted for any other degree or professional qualification.

Chapter 4 has been published (with minor modifications) in the following scientific publication:

St.Rose, S.G., Hunter, N., Matthews, L., Foster, J.D., Chase-Topping, M.E., Kruuk, L.E.B., Shaw, D.J., Rhind, S.M., Will, R.G. and Woolhouse, M.E.J. Comparative evidence for a link between Peyer's patch development and susceptibility to transmissible spongiform encephalopathies. *BMC Infectious Diseases* 2006 **6:**5

Suzanne Gail St.Rose

March 2006

To family, friends and all those
who made this work possible

Acknowledgements

Whether or not mentioned by name here, I am grateful to anyone who contributed to this dissertation. I would like to thank Mark Woolhouse, my principal supervisor, who has been a mentor and inspiration and who has provided immense guidance and support throughout the years. I am also grateful to Nora Hunter and Robert Will, my co-supervisors, for providing advice and useful comments on draft manuscripts over the past few years especially during busy times.

Many thanks go to Susan Rhind for her guidance and for assisting me in my initial studies on identification and quantification of Peyer's patches. Special thanks go out to Louise Matthews who assisted me with the mathematical side of things, and Margo Chase-Topping and Loeske Kruuk who provided me with (much appreciated) help in statistics. I am also grateful to Mike Miller for providing me with the CWD data for inclusion in this thesis.

I am indebted to numerous members of the NPU staff. Thanks to Jim Foster for organising my many farm visits, and Calum McKenzie for assisting me tremendously on the sheep farm. Also of invaluable help were Dawn Drummond and Dave Parnham who taught me several laboratory techniques including immunocytochemistry. Neil MacIntyre, thank you for your patience and for assisting me with the numerous photos and image analyses. I am also very grateful to Karen Brown and Patricia McBride, who assisted me with time-consuming dissections and gave me endless encouragement and to Moira Bruce who offered me helpful advice. Claudine Raymond and Bridget Glaysher provided me with useful advice and were supportive throughout the project. Thank you.

Special thanks to Wilma Robertson who assisted me immensely in retrieving numerous references.

To my family, and friends who are like family to me (like Simone Titus), thank you. You have been my rock throughout the years.

And to Marco. You have given me relentless support throughout the past years, and have taught me to appreciate what is different and unfamiliar. Thank you.

Table of Contents

List of Figures

List of Tables

Abbreviations

BSE	bovine spongiform encephalopathy
CJD	Creutzfeldt-Jakob disease
CNS	central nervous system
CI	confidence interval
CWD	chronic wasting disease
DNA	deoxyribonucleic acid
FAE	follicle-associated epithelium
FDC	follicular dendritic cell
FFI	fatal familial insomnia
FSE	feline spongiform encephalopathy
GALT	gut-associated lymphoid tissue
GPI	glycosylphosphatidyl inositol
GSS	Gerstmann-Sträussler-Scheinker syndrome
HR	hazard ratio
i/c	intracerebral
ICC	immunocytochemistry
IEL	intraepithelial T-lymphocyte
ILF(s)	isolated lymphoid follicle(s)
i/p	intraperitoneal
i/v	intravenous
kDa	kilodalton
LFV	lymphocyte-filled villi
LRS	lymphoreticular system
LSC	lymphohaemopoietic stem cell
LTβR	lymphotoxin β-receptor
LTβR-Ig	lymphotoxin β-receptor and human immunoglobulin
TNF-R1	tumour necrosis factor, receptor 1
MBM	meat and bone meal
M cells	microfold cells
mRNA	messenger ribonucleic acid
NPU	Neuropathogenesis Unit
nvCJD	new variant Creutzfeldt-Jakob disease
OR	odds ratio
PH	proportional hazard
PK	proteinase K
PP(s)	Peyer's patch(es)
PrP	protease-resistant protein/prion protein
PrP^{C}	cellular (host) protein
PrP^{d}	disease-associated prion protein
PrP^{res}	protease-resistant prion protein
PrP^{Sc}	protease-resistant prion protein /scrapie-specific prion protein
PrP^{sen}	protease-sensitive prion protein
RML	Rocky Mountain laboratory
RNA	ribonucleic acid
SAF	scrapie-associated fibrils
s/c	subcutaneous
SCID	severe combined immunodeficient mice
sCJD	sporadic Creutzfeldt-Jakob disease
SE	spongiform encephalopathy
SHa	Syrian hamster

TME	transmissible mink encephalopathy
TNF	tumour necrosis factor
TNF-R1	tumour necrosis factor-receptor 1
TSE	transmissible spongiform encephalopathy
UK	United Kingdom
vCJD	variant Creutzfeldt-Jakob disease

Abstract

The age distribution of the naturally occurring transmissible spongiform encephalopathies (TSEs) or prion diseases, including scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle and variant Creutzfeldt-Jakob disease in humans (vCJD) may be explained by age-dependent susceptibility to infection. Epidemiological studies have shown that exposure to BSE-contaminated meat and meat products alone cannot fully account for the young age distribution of vCJD cases, and that changes in susceptibility may also play a role. Oral exposure has been implicated as the most likely route of natural transmission of TSEs. Peyer's patches (PPs), part of the gut-associated lymphoid tissue (GALT), may represent a portal of entry for orally transmitted prions and appear to be the most likely sites of prion accumulation in the gastrointestinal tract. The main aim of this project was to determine whether the observed age-susceptibility relationship of scrapie, BSE and vCJD could be explained by the development of PPs in the gut. Statistical analyses were performed on an experimental scrapie dataset to determine whether there was an effect of age at exposure on scrapie outcome and incubation period of the disease, taking into consideration other potentially important factors related to susceptibility. PP tissue was quantified in the distal ileum of NPU Cheviot sheep, and data on measures of PP development in cattle and humans were extracted from previous studies. Anatomical PP data and estimates of age-related risks of infection, derived from mathematical models, were used to determine a potential link between age-related risk of natural TSE infection and the development of PP tissue in the three species. Because follicular dendritic cells (FDCs) have been implicated as the likely sites of prion replication in lymphoid tissues, immunocytochemistry was carried out to investigate the ontogeny of PrP-associated FDCs in ileal PP tissue in both mice and sheep. Results showed that age at exposure to the TSE agent is a potentially important factor in determining disease outcome as well as the incubation period of disease. Younger sheep were found to have shorter incubation periods than older animals following subcutaneous inoculation of the infectious agent, a finding which may be attributed to the development of peripheral nerves and which warrants further investigation. For sheep, cattle and humans, measures of PP development peaked in adolescent years followed by a decline, thereafter. There was a significant correlation between measures of PP development and estimated risks of natural TSE infection, with the two age-related distributions peaking in the same age group for all three species. Immunocytochemistry studies showed that PrP-associated FDCs can first be detected in 7 day-old mice, and provided further evidence for the presence of mature FDC networks in GALT of postnatal sheep. These findings imply that, in the absence of FDCs, mice younger than 7 days old may be less susceptible to oral scrapie challenge. The presence of FDCs in newborn sheep suggests that these animals may be susceptible to TSE infection in early postnatal life particularly at lambing when an infected placenta could act as a source of scrapie infection to the young lamb. Age-dependent susceptibility to oral TSE infection in mice and sheep may offer a convenient experimental framework with which to explore the reasons for age-dependent susceptibility to TSE infection in humans.


YOU GET A FREE CJD TEST WITH YOUR TENTH BURGER
GRE ZEE GRUB

Chapter 1: Introduction

1.1 Transmissible spongiform encephalopathies

Bovine spongiform encephalopathy (BSE) was first recognised in dairy cattle in the United Kingdom (UK) in 1985 but burst in on public awareness as "Mad Cow Disease" in 1986 (Wilesmith *et al.*, 1988). BSE belongs to a group of fatal, neurodegenerative disorders called the transmissible spongiform encephalopathies (TSEs) or prion diseases, which are widely recognised for their transmissibility. The terms TSE and prion disease have been used interchangeably throughout this thesis. Prion diseases involve modification of the normal, cellular host prion protein (PrP^{C}) into the abnormal, pathogenic isoform (PrP^{Sc}) (Prusiner *et al.*, 1998). The TSEs include, in animals, scrapie in sheep and goats, feline spongiform encephalopathy (FSE), transmissible mink encephalopathy (TME) and chronic wasting disease (CWD) in mule deer, white-tailed deer and elk (see Table 1.1). In humans, TSEs include kuru, fatal familial insomnia (FFI), Gerstmann-Sträussler-Scheinker syndrome (GSS) and Creutzfeldt-Jakob disease (CJD), of which there are sporadic, iatrogenic, familial and new variant forms (see Table 1.2).

The risk to humans from BSE fuelled much concern when, in 1996, a novel human disease affecting mainly young adults and then known as new variant CJD (nvCJD, now simply vCJD) was identified (Will *et al.*, 1996). During this time, previously unknown TSEs were also recognised in several other animal species including domestic cats (Wyatt *et al.*, 1991) and captive wild animals (Kirkwood and Cunningham, 1994; Willoughby *et al.*, 1992). Investigation of early cases of BSE suggested that protein supplements (meat and bone meal (MBM)) which were derived primarily from rendered cattle and which were fed to calves from 2 weeks to 6 months old, were a likely source of the disease (Wilesmith *et al.*, 1991). In the late 1970s there was a change in the rendering process used to produce MBM for livestock and feeding of this material to very young calves may have triggered the BSE epidemic (Horn, 2001). The MBM product, which was widely distributed throughout the UK, was infecting not only cattle but also other animals that were habitually being given this feed. These findings prompted speculation that the agent had crossed the species barrier (Bruce *et al.*, 1997). It has been recently shown that vCJD and BSE are caused by the same strain of agent (Bruce *et al.*, 1997), and that the outbreak of vCJD is almost certainly due to dietary exposure to BSE-contaminated beef and beef products (Bosque, 2002).

Table 1.1 Animal Transmissible Spongiform Encephalopathies

Disease	Host Species	Comments
Scrapie	Sheep, goats	First recognised as a distinct disease of sheep in the 18th century (Parry, 1983). Common in several countries throughout the world. Only Australia and New Zealand are certified as being free of the disease (Bosque, 2002).
Transmissible mink encephalopathy (TME)	Mink	First TSE to be identified in non-domestic animals (Bosque, 2002). Rare and largely confined to the United States (Marsh and Hadlow, 1992). Associated with feeding of scrapie-contaminated food (Marsh and Bessen, 1993). No evidence of TME occurring naturally in free-ranging animals (Williams and Miller, 2003).
Chronic wasting disease (CWD)	Mule deer White-tailed deer Elk	First recognised in captive mule deer in Colorado (Williams and Young, 1980). May be transmitted in deer and elk by direct contact or indirectly from the environment (Williams and Miller, 2002). Horizontal transmission likely to be important in sustaining CWD epidemics (Miller and Williams, 2003).
Bovine spongiform encephalopathy (BSE)	Cattle	First cases occurred in Great Britain in the early 1980s, and recognised as a prion disease in 1987 (Wilesmith *et al.*, 1988). Since recognition of BSE in 1986, over 180,000 cattle in the UK have developed the disease (Smith and Bradley, 2003). Suggested that BSE probably originated from sheep prions that acquired new characteristics after crossing into cattle, or represents a rare strain of scrapie or originated spontaneously in an individual cow (Bosque, 2002).
Feline spongiform encephalopathy (FSE)	Domestic cat	First identified in Great Britain in 1990. Believed to have been transmitted to cats through the consumption of BSE-contaminated feed (Ryder *et al.*, 2001).
TSEs in captive wild animals	Wild ruminants (antelope, Ankole cattle and American bison), wild cats (cheetahs, pumas, ocelots, tigers and lions).	Possibly due to consumption of BSE-contaminated feed (Kirkwood and Cunningham, 1994).

Table 1.2 Human Transmissible Spongiform Encephalopathies

Disease	Comments
Kuru	Found in Papua, New Guinea among the Fore-speaking people. Spread by ritual cannibalism (Gajdusek, 1977).
Gerstmann-Sträussler-Scheinker syndrome (GSS)	A rare variant of CJD first described in an Austrian family in 1936. Incidence of <0.1 per million per annum. An inherited disease. Age at onset: 35-55 years (Brown and Gajdusek, 1991).
Creutzfeldt-Jakob disease (CJD)	
i) Sporadic CJD (sCJD)	First detailed account of disease given in 1920 by Creutzfeldt. sCJD forms the majority of cases. Found worldwide but is rare (incidence of 1 per million per annum). Age at onset: 50-75 years (Brown and Gajdusek, 1991). Cause unknown.
ii) Familial CJD	Represents about 5-15% of the total CJD cases (Masters *et al.*, 1979). Related to presence of mutations of the prion protein (PrP) gene (Goldfarb *et al.*, 1992). Shows a somewhat younger age at onset than sporadic cases (Haltia *et al.*, 1979).
iii) Iatrogenic CJD	Transmitted through direct inoculation of contaminated material either centrally or peripherally, for example, via neurosurgery, dura mater implant, and growth hormone treatment (Brown *et al.*, 1992).
iv) Variant CJD (vCJD)	First documented in the UK in 1996 (Will *et al.*, 1996). Median age of onset is 28 years. Probably transmitted from cattle to humans through consumption of BSE-contaminated beef products. So far, only individuals with homozygous methionine (MM) genotypes have featured as (clinical) vCJD cases (Ironside and Head, 2004).
Fatal familial insomnia (FFI)	First reported in 1986. An inherited disease. Very rare.

Although vCJD cases are tragic at the individual level, the number of victims has remained reassuringly small from a public health perspective. However, since more than a million cattle with BSE may have entered the human food chain (Anderson *et al.*, 1996) and the incubation period may be as long as 40 years for vCJD (Baker *et al.*, 1998), the possibility of a significant epidemic of vCJD over the next 10-15 years exists. However, if the mean incubation period is short, with little variation from person to person, then most cases of vCJD may have already emerged.

What is most striking about the TSEs and what makes these diseases the subject of widespread interest is the apparent age distribution of cases (Figure 1.1). For instance, 80 per cent of scrapie cases die at age 2-4 years of age compared to only 3 per cent of cases that die at 7 or 8 years (Figure 1.1C) (Baylis *et al.*, 2004), and it is unclear as to why youth should be a risk factor for acquiring vCJD (Figure 1.1A). However, one explanation is related to the immune system of the young (Bosque, 2002). Various authors have shown, following experimental oral infection of rodents and mule deer fawns, that the initial stages of vCJD prion infection occur in the gut-associated lymphoid tissue (GALT) (Beekes and McBride, 2000; Sigurdson *et al.*, 1999). Whether the normal variation in the GALT bears any relation to susceptibility to TSE infection has so far remained unexplored, but it is tempting to speculate that such variations may predispose to (or protect from) TSE infection that is known to target these structures.

1.1.1 Characteristics of the TSEs

The TSEs are an unconventional group of diseases and share in common several unique characteristics, which distinguish them from other diseases. Prion diseases are known collectively as spongiform encephalopathies (SEs) because of the characteristic pathology they display in the central nervous system (CNS). Pathological changes include spongiform (vacuolar) degeneration, loss of neurons, astrocytosis, gliosis and in most TSEs, variable amyloid plaque formation (Brown *et al.*, 2000a; DeArmond, 1993; Detweiler and Baylis, 2003). Scrapie-associated fibrils (SAF) are consistently found in detergent-treated extracts of infected brain tissue examined by negative stain electron microscopy (Merz *et al.*, 1981). SAF have been observed only in scrapie and related SE diseases (Merz *et al.*, 1984; Somerville, 1985) and have never been found in diseases with other causes (Merz *et al.*, 1981). Unlike other infectious diseases, the TSEs are not accompanied by a detectable classical immune or inflammatory response (Carp *et al.*, 1985).

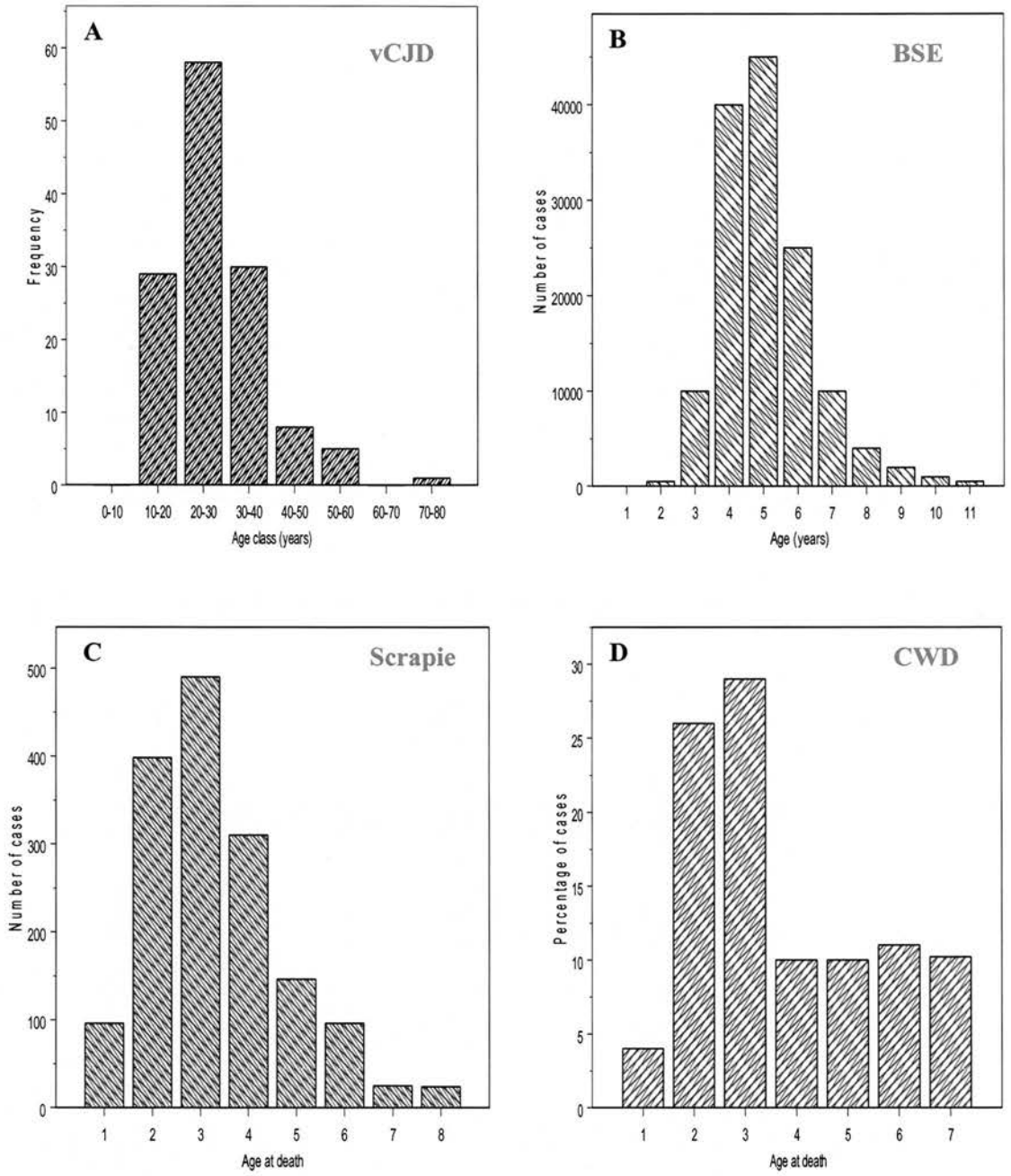

Figure 1.1 Age distribution of the naturally acquired TSEs. **A.** Age distribution of vCJD cases in British people. Data include 129 cases with onset of clinical disease before November, 2001 and comprises 71 men and 58 women (taken from Boëlle et al., 2004). **B**. Age at clinical onset of BSE in British cattle as of October, 2005 (taken from Defra, 2004). **C.** The frequency distribution of ages at death for scrapie cases (taken from Baylis *et al.,* 2004). **D.** Age distribution of CWD cases in captive mule deer in Colorado (data provided by M. Miller, 2005).

A striking feature of the causative agent is its extraordinary resistance to inactivation by physical and chemical treatments. The resistance of the causative agent was revealed when ten per cent of a group of Scottish sheep acquired scrapie after having been vaccinated against louping ill. The vaccine was prepared from a formalin-treated suspension of sheep brain extract, which was unknowingly contaminated with the scrapie agent (Gordon, 1946). The resistance of the agent is further exemplified by its ability to withstand ultraviolet irradiation, a finding, which suggests that the agent may be devoid of nucleic acid (Alper, 1992).

Sigurdsson (1954) first applied the term, slow viral infections, to the TSEs. This was based on the recognition that these diseases have unconventional characteristics, and prolonged incubation periods ranging from months to years (for example, one year for a mouse and four to six years for a cow). In humans, the disease type determines the age at onset of symptoms. For example, in sCJD and vCJD, symptoms become apparent at a mean age of 60 and 29 years, respectively. Prion diseases are progressive and debilitating with neurological symptoms, which inevitably lead to death (Lasmézas, 2003).

However, one characteristic, which distinguishes this group, whether sporadic, dominantly inherited or acquired by infection, is that most of the diseases listed (Table 1.1 and Table 1.2) have been successfully transmitted to experimental animals. Infectivity, for example, has been demonstrated by transmission of disease to experimental animals (typically mice or hamsters) by intracerebral (i/c) injection of infected material and subsequent serial passage to further mice and hamsters (Bruce *et al.*, 2002; Chandler and Fisher, 1963; Kimberlin *et al.*, 1989). Transmission via natural routes also occurs; in sheep and goats, scrapie can be naturally transmitted by infected dams to their offspring or from infected to uninfected members of a flock (Detweiler and Baylis, 2003). Because of their ability to be transmitted experimentally and/or naturally, these diseases have been appropriately termed the TSEs.

It is evident that from these unusual properties, the causative agent is very different from all other known plant and animal viruses, and also viroids. Because of the uncertainty surrounding the nature of the transmissible agent, several unorthodox hypotheses have been proposed.

1.1.2 The nature of the scrapie agent

Several hypotheses on the nature of the agent that causes TSEs have been proposed, involving every major class of macromolecules as possible components. Examples include: a "naked" nucleic acid genome without a protective protein coat (Diener, 1972); a small DNA virus (Kimberlin and Millson, 1967); a replicating polysaccharide (Field *et al.*, 1967); nucleic acid surrounded and stabilised by a polysaccharide coat (Adams and Field, 1967); a "holoprotein" which comprises a PrP^{Sc} component ("apoprion") and a nucleic acid component ("coprion") (the "unified theory") (Weissmann, 1991). The two fields of thought which have gained most credibility are the virino hypothesis (Bruce and Dickinson, 1987; Weissmann, 1991) and the prion or "protein only" hypothesis (Prusiner, 1991). Evidence for both is compelling and strongly defended.

The virino hypothesis is based on the concept of an unusual "sub-virus" particle, called a virino, which is composed of a non-coding nucleic acid genome that determines strain characteristics in tight association with a protective "structural" host-encoded protein (Dickinson and Outram, 1988) to which the infective agent would imprint an abnormal conformation through interference with normal metabolism.

The hypothesis explains many of the unconventional properties of the infectious agent, such as the difficulty in purifying the agent, its resistance to ultra-violet radiation and an absence of an immunological response in infected animals. The host origin of the protein may explain the difficulties which arise when trying to separate the infectious agent from the host material (Hunter, 1972; Millson and Manning, 1979; Prusiner *et al.*, 1981). The low molecular weight of the nucleic acid could also contribute to difficulties in isolating and detecting nucleic acid in partially purified preparations (Carp *et al.*, 1985), and may offer an explanation for the remarkable resistance of the scrapie agent to irradiation (Alper *et al.*, 1978). Binding of the agent genome to host protein would provide the resistance to inactivation for which the agent is renowned, and render it "immunologically invisible" to the host (Dickinson and Outram, 1988). The infectious nature of such a particle may be dependent on the continued interaction between the nucleic acid and the protein coat. Hence, if the genome exists as an unprotected nucleic acid, infectivity could be lost (Bennett *et al.*, 1992) or could become difficult to detect.

The hypothesis attempts to explain the variability of scrapie isolates when passaged in rodents (Dickinson and Fraser, 1977), and the occasional strain phenotypic changes

(mutations) which occur spontaneously during passage (Bruce and Dickinson, 1987; Kimberlin *et al.*, 1989). Such phenotypic features have also been observed with isolates from other TSEs, such as CJD and Kuru (Gibbs *et al.*, 1979).

Aiken and Marsh (1990) have reviewed several techniques used to identify nucleic acids unique to the scrapie agent. Hence, although the idea of a virion remains intellectually attractive, no credible evidence for an agent-specific nucleic acid has yet been forthcoming.

Griffith (1967), in light of the previous finding that the infectious agent was too small to possibly be a virus or any other known type of infectious agent, proposed that the transmissible agent was a protein that had the ability to self-replicate in the host. This marked the beginning of the so-called "protein-only" or "prion" hypothesis of TSE propagation, and represented the first clash with the biological dogma that conventional pathogens are unable to replicate in the absence of nucleic acid.

1.1.3 Prion protein (PrP^{Sc})

Since the 1980s, Stanley Prusiner's group has led research supporting the "protein only" hypothesis of TSE transmissibility. Prusiner's prion hypothesis is based on two major points: (i) there is no demonstrable nucleic acid in infectious PrP^{Sc} preparations and (ii) the scrapie agent is highly resistant to harsh procedures (Bolton *et al.*, 1984). He first coined the term "prion" (to distinguish the scrapie agent from viruses and viroids), which he defined as, "a small proteinaceous infectious particle that resist inactivation by procedures which modify nucleic acids", and suggested that PrP^{Sc} (or scrapie-associated prion protein) is the sole component of the prion (Prusiner, 1982). It was proposed that PrP^{Sc}, when introduced into a normal cell, causes the conversion of PrP^{c} (or normal host cellular prion protein) or its precursor into PrP^{Sc} (Prusiner, 1991).

PrP^{Sc} is the only characterized macromolecule found in purified preparations of scrapie agent (Diringer *et al.*, 1983; Prusiner, 1991); no specific nucleic acid has been detected in highly purified PrP^{Sc} preparations (Kellings *et al.*, 1992; Meyer *et al.*, 1991) despite long-standing efforts. Furthermore, infectivity was convincingly neutralised by agents that destroy protein structure and, more importantly, by anti-PrP antibodies (Prusiner, 1982). The prion hypothesis is also supported by genetic studies, which have shown that most, if not all, cases of human familial SEs are linked to mutations in the PrP gene (Hsiao *et al.*, 1989; Ironside, 1996), and that mice expressing PrP genes with point mutations linked to GSS spontaneously

develop neurological dysfunction, spongiform brain degeneration and astrocytic gliosis (Hsiao and Prusiner, 1991; Kaneko *et al.*, 2000). Other studies have shown that transgenic mice devoid of the PrP gene and lacking PrP^C, are resistant to scrapie infection, developing neither symptoms of scrapie nor allowing propagation of the infectious agent (Büeler *et al.*, 1992). The latter result demonstrated that PrP^C was required for disease propagation. Another strong argument in favour of the prion hypothesis was based on a seminal experiment by Prusiner and his colleagues, which showed that the species barrier can be overcome by introducing a Syrian hamster (SHa) PrP transgene into the recipient mouse line (Prusiner *et al.*, 1990; Scott *et al.*, 1989). Hence, when inoculated with hamster prions, some strains of mice containing SHa PrP transgenes had a short incubation period of 75 days, whereas, in the non-transgenic controls, the incubation period was about 500 days (Scott *et al.*, 1989). Furthermore, prions generated from such transgenic mice are highly infectious to the hamster but not to the mouse. The same transgenic mouse strain, infected with mouse-derived prions, yields preparations highly infectious for mice but not for hamsters (Prusiner *et al.*, 1990). These experiments show that hamster PrP^C and not murine PrP^C is a suitable substrate for conversion to hamster PrP^{Sc} by hamster prions and vice versa.

The prion concept has been largely developed from the fact that infectivity in hamster brain homogenates is resistant to a variety of treatments expected to destroy nucleic acid but some of the infectivity is abolished only by certain protein-specific agents (Prusiner *et al.*, 1984). All conventional viruses normally depend on a protein coat for their integrity and infectivity. Interference with the protein coat will consequently lead to destruction of the virus. Hence, it is accurate to assume that the TSE genomic material is protected by a protein coat as is observed in viruses.

The idea that scrapie could be caused solely by an infectious protein still remains far-fetched and unconvincing for many scientists. The most common arguments used against the protein-only hypothesis of prion transmissibility are the existence of several strains of the agent (Bruce and Dickinson, 1987), and the fact that infectivity is not strictly associated with PrP^{Sc} in some situations (Lasmézas *et al.*, 1997). However, it has been suggested, based on accumulated evidence, that "prion strains" are the result of the existence of multiple PrP^{Sc} conformations, which provide a specific template for the faithful conversion of the host protein into the disease-inducing conformer. Furthermore, conformation-dependent assay is one PrP-based method used to distinguish between different "prion strains", and exploits the

fact that discrete PrP epitopes can be differentially recognised by the antibodies used in the assay depending upon the conformation of the abnormally folded protein (Safar *et al.*, 1998).

Under certain experimental conditions, wild-type mice inoculated with BSE (Lasmézas *et al.*, 1997) and transgenic mice inoculated with human TSEs (Collinge *et al.*, 1995; Manson *et al.*, 1999) developed a TSE-like disease but PrP^{Res} (abnormal disease-associated or protease resistant prion protein) was not detected. The interpretations that stem from these observations are that the pathogenic, misfolded PrP is not necessarily protease-resistant, or that the infectious agent comprises a hitherto undiscovered molecule (presumably but not necessarily a nucleic acid) in addition to PrP (Lasmézas, 2003).

1.1.3.1 Prion protein is encoded by the PrP gene

In 1982, Stanley B. Prusiner achieved a 100-fold purification of the scrapie agent from SHa brains and for the first time, showed that infectivity of the agent depended upon PrP (Prusiner, 1982), an evolutionary conserved glycoprotein (Bosque, 2002). The PrP gene is expressed in both normal and scrapie-infected brain tissue, but can also be found in other tissues both within and outside the CNS (Bendheim *et al.*, 1992). In hamsters and mice, PrP messenger ribonucleic acid (mRNA) is most abundant in brain tissue (Chesebro *et al.*, 1985) especially in neurons (Prusiner, 1991). The cognate complementary deoxyribonucleic acid (DNA) and gene were cloned, based on the partial sequences of PrP and were found to be encoded by a single, host gene, PrP (Basler *et al.*, 1986; Oesch *et al.*, 1985) on the short arm of chromosome 20 in humans (Sparkes *et al.*, 1986).

This led to the recognition of two isoforms of PrP, the normal cellular form (PrP^{C}) and the abnormal disease-associated or resistant form (PrP^{Res}), which represents an abnormal structural conformation of PrP^{C} (Pan *et al.*, 1993). The amino acid chain of PrP, which, in the hamster, is composed of 254 amino acid residues (Bennett *et al.*, 1992) folds into a specific three-dimensional structure that is presumably essential to PrP function (Oesch *et al.*, 1985). The entire open reading frame, that part of a gene coding for a protein, is in a single exon, ruling out the possibility that PrP^{C} and PrP^{Sc} differ as a result of alternative mRNA splicing (Stahl and Prusiner, 1991).

PrP^{C} and PrP^{Res} are distinguished by their physical properties. PrP^{C} is a 33-35 kilodalton (kDa) glycoprotein anchored to the cell surface by a glycosylphosphatidyl inositol (GPI) moiety (Aguzzi and Weissmann, 1997; Oesch *et al.*, 1985; Prusiner, 1991) and its structure

comprises approximately 43% α-helix and 3% β-sheet (Riek *et al.*, 1997). On proteinase K treatment, it was found that while 33-35 kDa PrP from an uninfected animal was completely hydrolysed, the 33-35 kDa PrP derived from infected animals was partially degraded to PrP^{Res} that corresponded to 27-30 kDa PrP, without loss of infectivity (Oesch *et al.*, 1985). PrP^{Sc} retains 34% α-helix but incorporates a greatly increased amount of β-sheet (Pan *et al.*, 1993). Due to its *sen*sitivity to protease digestion, PrP^{C} it is also known as PrP^{Sen}. On the other hand, due to its partial *res*istance to protease treatment, PrP^{Sc} is sometimes referred to as PrP^{Res}. PrP^{Res} is usually represented as PrP with a superscript, which indicates the disease in question, for example, PrP^{Sc} for scrapie in sheep and PrP^{BSE} for BSE in cattle. In vitro, PrP 27-30 forms into rod-like particles (unlike PrP^{C}), and is able to bind to Congo red dye, which reveals green birefringence in polarized light. PrP 27-30 specific antibodies indicate that the amyloid plaques, which develop in scrapie-infected brains, also contain protease-resistant PrP (Bendheim *et al.*, 1984; DeArmond *et al.*, 1987), as do the amyloid plaques seen in cases of CJD, GSS and kuru (Guiroy *et al.*, 1994).

1.1.4 The role of PrP in uninfected animals

PrP^{C} plays a pivotal role in the acquisition of prion diseases in animals and humans, although its exact physiological role has not yet been elucidated. The molecule is expressed predominantly within the CNS (Aguzzi and Weissmann, 1997; Prusiner, 1991), but is also found in several non-neuronal tissues as demonstrated by the use of antisera to the protein and ribonucleic acid (RNA) detection methods (Chesebro *et al.*, 1985; Robakis *et al.*, 1986). PrP^{C} has been shown to be present in various regions of the hamster brain, including the cortex, hippocampus, striatum, olfactory bulb, hypothalamus, midbrain, cerebellum and brainstem (Bendheim *et al.*, 1992), although various authors have found some regional differences in the abundance and localization of PrP^{C} (DeArmond *et al.*, 1987). The cellular isoform of PrP has also been detected on the surface of lymphocytes and activated T-lymphocytes in humans and mice (Cashman *et al.*, 1990; Mabbott *et al.*, 1997) and at higher levels on follicular dendritic cells (FDCs) of lymphoid tissues (McBride *et al.*, 1992). Northern blot and/or Western blot have been used to detect PrP^{C} in a number of peripheral tissues including heart, lung, pancreas, testes and kidney in the rodent (Oesch *et al.*, 1985; Robakis *et al.*, 1986) as well as skeletal muscle and uterus in sheep (Horiuchi *et al.*, 1995). PrP^{C} has been detected on epithelial cells of skin and mucous membranes suggesting that these cells may well represent possible first targets for peripheral infection with the agent (Pammer *et al.*, 1998). In peripheral organs, the identification of PrP^{C}-expressing cells is

much less documented than in the brain. The association with various cell types exhibiting different physiological functions suggests that PrP^C may have several different roles.

In an attempt to unveil the function of PrP^C, PrP^C knock-out mice (variously termed PrP nulls, $PrP^{0/0}$ or $PrP^{-/-}$ mice) were used but no obvious phenotypic deficits were observed (Büeler *et al.*, 1992). Although PrP null mice breed and behave normally, they exhibit a lack of fine tuning of neuronal functions including aberrant sleep patterns (Tobler *et al.*, 1996) and increased locomotor activity (Roesler *et al.*, 1999) as well as a reduced T-lymphocyte response (Mabbott *et al.*, 1997). One study showed that, although PrP null mice grew normally after birth, at about 70 weeks of age all began to show progressive signs of ataxia (Sakaguchi *et al.*, 1996). However, alternative explanations have been offered for this ataxia, which appears to be due to up-regulation of a related gene or the Doppel gene (Moore *et al.*, 1999), rather than a lack of PrP expression. PrP null mice were found to harbour lower copper concentrations in synaptosomal fractions than their PrP^C expressing counterparts. This suggests that PrP^C could regulate copper concentration in the synaptic region of the neuron and may play a role in the re-uptake of copper into the presynaptic cell (Kretzschmar *et al.*, 2000).

Several experimental findings suggest that PrP^C may play a role in signalling, cell survival and differentiation. Binding of PrP^C to signalling molecules suggests a role as a transmitter of information from the extracellular environment to the cell and a trigger for a molecular cascade (Mouillet-Richard *et al.*, 2000). Moreover, the presence of the protein in fibre tracts of elongating axons of hamster brain, as well as at the surface of elongating retinal axons (Sales *et al.*, 2002), is suggestive of a signalling role of PrP^C during axonal growth. PrP^C may play a major role in neuroprotective signalling. Mice expressing N-terminally truncated PrP^C in a $PrP^{0/0}$ genetic background suffered from neurodegeneration, and the normal phenotype was rescued by the expression of the full-length protein (Shmerling *et al.*, 1998). In contrast to these findings, some studies have shown that over-expression of PrP^C sensitizes cultured cells to apoptotic death (Paitel *et al.*, 2002), and abnormal accumulation of cystolic PrP^C results in neurodegeneration and acute toxicity (Ma and Lindquist, 2002; Ma *et al.*, 2002). In the light of present knowledge, it seems that although PrP^C is involved in cell survival, any abnormal deviation from its steady state concentration and compartmentalization in the cell results in the protein exerting adverse cytotoxic effects. In summary, although the role of PrP^C remains unclear, its importance lies in providing useful insight into the pathogenesis of TSE disease.

1.1.5 The role of PrP in TSE pathogenesis

PrP plays a pivotal role in control of development of TSEs. Natural prion infection may result through ritualistic cannibalism as in kuru, or through the ingestion of prion-contaminated meat and bone meal as in BSE. However, the exact size and physical nature of the TSE agent is not known, and this hampers understanding of the likely mechanisms and rate of uptake of the agent following oral infection. Furthermore, it is not known whether infectious particles of a different size are required to cause disease when administered by the oral compared with the i/c routes. Although the pathological form of PrP is usually found in multimeric fibrillar aggregates in the brain of TSE-affected individuals, it is not known whether food preparation and subsequent processing during digestion may release a monomeric form of PrP^{Sc} (Shmakov and Ghosh, 2001). The infectious prion particle is generated through the conversion of endogenous PrP^{C} to PrP^{Sc}, the latter providing a template that promotes the conversion of PrP^{C} and the conversion being catalysed by the pathogenic agent (Prusiner, 1991). In the heterodimerisation model, the activation energy barrier at each new structural transformation is lowered promoting the conversion of PrP^{C} to PrP^{Sc}. In the case of familial human SEs, PrP mutations would destabilise PrP^{C} thereby, lowering the barrier and facilitating the conversion of PrP^{C} to PrP^{Sc}. In sporadic prion diseases, somatic mutations might destabilise PrP^{C} promoting its conversion into PrP^{Sc} (Huang *et al.*, 1996). The PrP^{Sc} molecules would eventually aggregate, a process which may be facilitated by an unidentified molecular chaperon, referred to as Factor X (Telling *et al.*, 1995). Whether or not PrP^{Sc} itself is responsible for disease manifestation remains a major subject of controversy.

1.1.5.1 Experiments using knock-out and transgenic mouse models

PrP knockout and transgenic mouse models have been used extensively to study TSE pathogenesis. Mice have been experimentally produced in which the PrP gene has been deleted. The advent of these so-called "knock-out" mice ($PrP^{0/0}$ or mice homozygous for the disrupted PrP gene) and inoculation experiments in these hosts have supported the concept that PrP is indispensable for disease induction by TSE agents. Büeller *et al.* (1993) have shown that when $PrP^{0/0}$ and $PrP^{+/+}$ (wild-type counterpart) mice were inoculated intracerebrally with a high dose of the Chandler isolate of mouse-adapted prions (Chandler, 1961), $PrP^{+/+}$ controls died within about 180 days, while $PrP^{0/0}$ mice, devoid of PrP^{C}, were completely protected against scrapie and showed normal development and behaviour up to 13 months after inoculation. Although $PrP^{0/0}$ mice were shown to harbour small amounts of infectivity for up to two days post-inoculation, the most likely explanation for these findings

was the persistence of inoculum (Sailer *et al.*, 1994). Manson and colleagues (1994) have also shown that inoculation of $PrP^{0/0}$ mice with TSE-infected tissue failed to produce any signs of disease either clinically or pathologically.

Various authors (Brandner *et al.*, 1996; Blätter *et al.*, 1997) have re-introduced PrP into $PrP^{0/0}$ mice in the form of brain grafts from transgenic mice over-expressing PrP. After i/c inoculation (Brandner *et al.*, 1996), grafts were found to have accumulated high levels of PrP^{Sc} and infectivity. Furthermore, grafts showed the severe histopathological changes characteristic of scrapie, and substantial amounts of graft-derived PrP^{Sc} had migrated into the host brain. Despite this, the adjacent host brain tissue (devoid of PrP^{C}) underwent no pathological changes after a 16-month period.

Blättler *et al.* (1997) showed that PrP-expressing neurografts in $PrP^{0/0}$ mice do not exhibit histopathological changes after intraperitoneal (i/p) or intravenous (i/v) inoculation with scrapie prions. Furthermore, peripheral (i/p or i/v) inoculation did not produce scrapie pathology in mice with reconstituted lymphohaemopoietic systems with PrP-expressing cells. The results of these two studies show that PrP is essential for the development of pathology and spread of infection both within the CNS, and from the peripheral tissues to the CNS.

Experiments with transgenic mice expressing various levels of homologous or heterologous PrP have shown that the level of expression of the PrP gene influences susceptibility to the prion diseases (Büeler *et al.*, 1993). Mice carrying a single PrP allele (heterozygous $PrP^{0/+}$ mice) are found to be partially protected from scrapie when inoculated with mouse-adapted prions (Büeler *et al.*, 1993). Furthermore, disease incubation period in $PrP^{0/+}$ mice was found to be noticeably longer than wild-type ($PrP^{+/+}$) mice. The fact that $PrP^{0/+}$ heterozygous mice exhibit considerably longer incubation times compared to their wild-type counterparts (and PrP expression appears to be positively correlated with disease susceptibility) suggests that PrP^{C} concentration may be an important rate-limiting factor in determining the progression of the disease (Büeler *et al.*, 1993).

Because transgenic "null" mice are resistant to TSE infection and appear to be clinically normal, this has led to the suggestion of breeding animals without PrP genes (through ablative gene therapy, or the use of anti-sense oligonucleotides to "switch off" PrP production) in order to produce prion-disease-resistant animal strains which are safe for

human consumption (WHO, 1999). The possibility of gene therapy for human prion diseases also exists (WHO, 1999). However, the dilemma lies in determining the normal function of PrP^C and whether or not critical cell functions are dependent on it. Until the role of PrP^C becomes established, this possibility will remain a likely conjecture.

1.1.6 TSE susceptibility in animals and man

The primary risk factor associated with scrapie is explicitly the PrP genotype. However, genetic analyses of scrapie incubation period and susceptibility to natural scrapie are complex because of uncontrolled variables such as the time and extent of exposure and the strains of scrapie agent involved. The genetic control of TSEs in different species and the complexity arising as a result of host-strain interactions and other risk factors associated with the incubation period and susceptibility to TSEs are described in this section.

1.1.6.1 Genetic control of TSEs

1.1.6.1.1 Genetic control of scrapie in sheep

The susceptibility/resistance of sheep to scrapie is largely under genetic control (Hunter *et al.*, 1997), and this has formed the basis for the National Scrapie Plan in which sheep are specifically bred to prevent scrapie developing (Eglin *et al.*, 2005). The Cheviot sheep flock (Neuropathogenesis Unit (NPU) Cheviots), which was founded by Alan Dickinson in 1960, has made a considerable contribution to the understanding of host genetic factors involved in scrapie susceptibility. Dickinson (1976) selected two lines of sheep, a positive and a negative line, depending on the incubation period in the animals following injection with a source of scrapie called SSBP/1. Cheviots that developed disease from subcutaneous (s/c) inoculation in 150-400 days were bred as the positive line and those, which showed no clinical signs of disease within their normal lifetime, were grouped in the negative line. The gene, which controls the experimental scrapie incubation period in these sheep, was called *Sip* (for *s*crapie *i*ncubation *p*eriod), and has two alleles sA and pA (Dickinson and Outram, 1988). Negative-line Cheviots were described as Sip^{pApA} homozygotes whilst positive-line Cheviots were Sip^{sAsA} homozygotes or Sip^{sApA} heterozygotes (Dickinson, 1976) (Sip^{sApA} being partially dominant (Foster and Hunter, 1991)). The behaviour of Sip^{sA} and Sip^{pA} incubation period alleles can only be defined in terms of infecting strain of scrapie agent and the recipient breed of sheep. For example, SSBP/1 has shorter incubations in Sip^{sA} carriers (positive-line) than in Sip^{pApA} (negative-line) Cheviots. However, for CH1641 and BSE strains, the incubation phenotypes of the *Sip* alleles are reversed, with Sip^{pApA} homozygous sheep having shorter incubations than positive-line animals (Foster and Dickinson, 1988a; Foster and

Dickinson, 1988b; Foster *et al.*, 1993). CH1641 and BSE differ from each other in many respects and are not related (Hunter, 2003).

The *Sip* gene is very closely linked and most probably identical to the PrP gene (Hunter, 1997a). Molecular techniques, including the use of polymerase chain reaction, restriction enzyme digestion, allele specific amplification, denaturing gradient gel electrophoresis and sequencing have led to the discovery of many polymorphisms in the PrP gene of sheep. The three main amino acid substitutions that play a significant role in both experimental and natural scrapie onset are at codons 136, 154 and 171 of the PrP gene (Hunter, 1997a). At codon 136, the amino acid specified can be either valine (V) or alanine (A); at codon 154, it is arginine (R) or histidine (H); and, at codon 171, arginine (R), glutamine (Q) or histidine (H). These three amino acid substitutions define five major PrP alleles, namely, A_{136}, R_{154}, Q_{171}, VRQ, ARH, AHQ and ARR (Table 1.3). A genotype can be written simply as VRQ/ ARQ. As many as 12 different sheep PrP allelic variants based on the three codons have been described (Hunter, 2003). However, only 5 alleles (mentioned above) are predominantly seen in sheep in many studies worldwide (Belt *et al.*, 1995). The frequency and distribution of the PrP allelic variants differ from breed to breed. For example, the VRQ allele is rare in Suffolk sheep but not in Texel, Swifter and NPU Cheviot sheep, and the ARH allele is common in Texel sheep but rare in most other breeds (Hunter, 1997a).

Table 1.3 PrP polymorphisms in sheep

Allele[a]	Nucleotide sequences[b]	Phenotypic effect
ARQ	GCC...CGT...CAG	Associated with susceptibility in some breeds[c]
VRQ	G**T**C...CGT...CAG	Associated with susceptibility
ARR	GCC...CGT...C**GG**	Associated with resistance
ARH	GCC...CGT...CA**T**	Usually associated with resistance
AHQ	GCC...C**AT**...CAG	Usually associated with resistance

[a]Amino acids specified by codons 136, 154 and 171, respectively.

[b]Bold type indicates nucleotide sequence difference from ARQ.

[c]ARQ homozygotes are normally susceptible in Suffolks (which lack the VRQ allele) and in Romanovs and Icelandics (which have the VRQ allele) but not normally in Cheviots (which also have the VRQ allele).

(Taken from Woolhouse *et al.*, 2001).

In general, polymorphisms at codons 136, 154 and 171 are associated with scrapie susceptibility. In many breeds, such as Cheviot, susceptibility to scrapie is linked to valine at

codon 136, with VRQ/VRQ homozygotes being the most susceptible genotype (Hunter *et al.*, 2000). For example, affected NPU Cheviots with a gene/dose effect where 77% of the natural scrapie cases were VRQ/VRQ and 23% were ARQ/ARQ, died at a mean age of 907 and 1462 days, respectively (Hunter *et al.*, 1996). Certain PrP gene alleles offer a protective effect, for example, ARR, so that animals with the VRQ/ARR genotype have only marginal susceptibility and rarely develop natural scrapie (Hunter *et al.*, 1996; Hunter *et al.*, 1997). ARR sheep are generally considered to be clinically resistant to natural scrapie with the exception of one reported case in Japan (Ikeda *et al.*, 1995), and recently reported cases in Norwegian sheep (Le Dur, 2005).

This observation may mean that these so-called atypical cases might be genetically different and that scrapie strains different from those in Western Europe may exist (Laplanche, 1999). Although it has been reported that three ARR/ARR sheep became infected (all with extensive incubation periods) after being injected intracerebrally with BSE-infected bovine brain homogenate (Houston and Gravenor, 2003), this result does not necessarily imply that ARR/ARR sheep are naturally susceptible to BSE. However, recent evidence indicates that ARR/ARR sheep may harbour subclinical infection (González *et al.*, 2005). Although the National Scrapie Plan was initiated to eventually eradicate scrapie (as well as any BSE in sheep) by increasing the frequency of the ARR allele and decreasing the frequency of the VRQ allele (Houston *et al.*, 2003) on the basis of initial observations, these new findings may call for a reappraisal of this strategy in the long term.

The present view is that although the PrP genotype is the primary risk factor associated with scrapie, it is not a purely genetic disease. Hunter and colleagues (1997) have described two healthy Cheviot sheep, aged 36 and 96 months, carrying the VRQ allele in Australia, a scrapie-free country. This observation implies that scrapie is not necessarily a spontaneous genetic disease but most likely results from an exogenous pathogenic agent (Hunter *et al.*, 1997). ARR/ARR sheep are susceptible to i/c injection with BSE, which suggests that these animals do not have complete genetic resistance to prion infection (Houston and Gravenor, 2003). Foster and colleagues (1996) have shown that transmission of scrapie can be prevented or development of the disease delayed in some genetically susceptible sheep. It is possible that other polymorphisms in the PrP gene and other undefined genes could modulate sheep susceptibility to scrapie and explain perhaps why some animals with susceptible genotypes do not develop disease (Diaz *et al.*, 2005; Laplanche *et al.*, 1999). During the perinatal period, lambs in a flock with a high incidence of natural scrapie are exposed to

greater levels of infectivity. Studies should take into account that several biological factors such as development and physiology of an animal's immune system and age at exposure may also come into play when determining susceptibility to prion infection. This possibility should not be overlooked. In natural scrapie other uncontrolled variables including time of exposure, different levels of infectivity pressure in contaminated environments and scrapie strain appear to be necessary for development of scrapie and make genetic analysis of scrapie incubation and/or susceptibility complex (Foster and Dickinson, 1988a). These variables will be discussed later.

The incubation time for the same prion isolate may be different in distinct mouse strains and is determined predominantly by the PrP gene (Carlson *et al.*, 1986; Hunter *et al.*, 1987). Short and long incubation periods are associated with two distinct PrP alleles, Prn-p^a (which produces the PrP A allotype) and Prn-p^b (which produces the PrP B allotype), respectively. The *a* and *b* alleles of PrP differ at codons 108 (Leu/Phe) and 189 (Thr/Val) (Carlson *et al.*, 1986). These PrP alleles are analogous to the two alleles of the *Sinc* gene, s7 and p7, identified by Dickinson and others (1968). The host PrP genotype does not account for all of the variation seen in the incubation period, indicating that other genes are probably involved in the control of the disease (Manolakou *et al.*, 2001; Moreno *et al.*, 2002), although little is known of their nature and function.

1.1.6.1.2 Cattle and elk **PrP** *gene polymorphisms*

In cattle, only one specific polymorphism (a 23-bp insertion/deletion polymorphism in the putative PrP promoter region) in the bovine PrP gene has so far, been associated with BSE susceptibility (Sander *et al.*, 2004). Although a variable number of octarepeats has been reported in bovine PrP with the majority of cattle having five octarepeats instead of six (Goldmann *et al.*, 1991), it is unlikely that the repeats affect the incidence of BSE (Hunter *et al.*, 1994; Neibergs *et al.*, 1994).

In Rocky Mountain elk, MM 132 homozygosity appears to predispose exposed elk to CWD (O'Rourke *et al.*, 1999). The elk is the first non-human species with a reported polymorphism at the corresponding site (O'Rourke *et al.*, 1998). Heterozygosity does not appear to be associated with prolonged incubation time since two of the CWD-affected methionine/leucine elk were diagnosed at 2 years of age (O'Rourke *et al.*, 1999).

1.1.6.1.3 PrP polymorphisms in human TSEs

Susceptibility of humans to vCJD is also linked to PrP polymorphisms. All patients with vCJD reported so far, are homozygous for the amino acid methionine (M) at position 129 (Will, 2003), with the exception of one patient with the MV (methionine/valine) genotype thought to be infected via blood transfusion (Peden and Ironside, 2004). Molecular genetic studies have also shown that multiple mutations in the PrP gene on chromosome 20p, which codes for the prion protein, are associated with familial CJD, GSS disease, and fatal familial insomnia (Goldfarb and Brown, 1995). Studies have shown that the MV polymorphic variation at position 129 of the PrP gene affects the age at onset and duration of illness in familial TSE (Baker *et al.*, 1991; Dlouhy *et al.*, 1992) and influences susceptibility to the sporadic and iatrogenic forms (Brown *et al.*, 2000b; Collinge *et al.*, 1991). Kuru, which has some phenotypic similarity to vCJD (Lantos *et al.*, 1997) and a common mode of transmission (kuru and vCJD are the only human TSEs transmitted naturally), preferentially affects individuals with the PrP 129 MM genotype (Lee *et al.*, 2001), and homozygosity (MM) appears to be associated with an earlier age at onset and a shorter duration of illness (Cervenakova *et al.*, 1998; Lee *et al.*, 2001). Since the codon 129 genotype has a marked influence on susceptibility to kuru and vCJD, cases of human BSE infection with a valine homozygous (VV) or heterozygous (MV) codon 129 genotype may yet occur (Will, 2003) and could have longer incubation periods, as suggested by recent studies of kuru (Goldfarb *et al.*, 2004; Huillard d'Aignaux *et al.*, 2002). Hence, further cases of vCJD may occur in older individuals with alternative 129 genotypes, signalling a maturing evolution of the vCJD "epidemic" (Lee *et al.*, 2001). Three large-scale studies testing tonsil and appendix tissues are currently underway in the UK to determine the prevalence of asymptomatic infection (Hilton *et al.*, 2002), however, the unknown sensitivity and specificity of the tests at different stages of the incubation period will necessitate careful interpretation of any results (Ghani *et al.*, 2003).

Like scrapie, the PrP gene may not solely explain susceptibility to vCJD since about 38 per cent of the UK population carry the high-risk methionine 129. One study investigated the distribution of codon 129 polymorphisms in patients in France and the UK who had acquired CJD iatrogenically by human growth hormone (Brandel *et al.*, 2003). UK patients had a low proportion (4%) of the MM genotype compared to French patients (62%) and this discrepancy was attributed to probable infection with different strains of the CJD agent in human growth hormone in France and the UK. This is possible since different host-strain interactions exist under experimental conditions. For example, ARR/ARR sheep have never

been orally infected with BSE (Kao *et al.*, 2003), and humans with MV or VV codon 129 genotypes appear to be so far, resistant to oral infection of the BSE strain of prion (Brandel *et al.*, 2003).

The relatively small number of vCJD cases diagnosed to date and the many biological and epidemiological uncertainties surrounding this novel disease make future projections of the epidemic difficult. Recent results from a study to detect abnormal prion protein in UK tonsil and appendix specimens (Hilton *et al.*, 2004) suggest a prevalence of 237 cases per million for prion infection, which implies that the extent of vCJD infection in the UK population is far greater than current clinical cases of vCJD would suggest. A number of subclincial or preclinical infections owing to the effect of different genotypes may be the cause of this discrepancy (Clarke and Ghani, 2004). While all clinical cases to date have been identified as MM, it is possible that other genotypes of individuals exposed orally to BSE, will develop disease with longer incubation periods. Support for this hypothesis comes from the recent identification of infection in the spleen of a patient with the MV genotype believed to be infected via blood transfusion (Peden and Ironside, 2004). A high incidence of subclinical infection has been identified in animal experiments using MM transgenic mice that have been inoculated with the BSE agent (Asante *et al.*, 2002). If there are two subpopulations of vCJD patients, one with "short" incubation times and methionine homozygosity, the other with "long" incubation times and valine homozygosity or heterozygosity, one would expect that in the next couple of years, the age distribution of vCJD cases will become bimodal and the proportion of older patients will increase (Valleron *et al.*, 2001). So far, the age-distribution of vCJD cases has not shifted.

1.1.6.2 Strain of agent, dose and route of infection

Host PrP genotype does not account for all of the variation seen in the incubation period, and not all genetically susceptible sheep develop scrapie when orally challenged or when residing in naturally infected flocks (Foster *et al.*, 2001; O'Rourke *et al.*, 1997). Scrapie occurs occasionally in animals which have one ARR allele (in the genotype ARQ/ARR or VRQ/ARR) (Hunter, 1997a), and naturally infected Suffolk sheep flocks do not demonstrate 100 percent scrapie infection of susceptible genotypes (Jeffrey *et al.*, 2001b). One study showed that in field outbreaks of scrapie or following oral dosing, between 12 and 20 percent of susceptible genotypes do not develop the disease (Jeffrey *et al.*, 2001b). These observations imply that there may be other factors involved in the control of the disease such as additional genetic factors (Hunter *et al.*, 1996), environmental factors (Healy *et al.*, 2004),

variable conditions for oral exposure (such as rumen contents) (O'Rourke *et al.*, 1997), age at infection (Diaz *et al.*, 2005) and agent strain (Goldmann *et al.*, 1994).

Host strain interactions are well documented in TSE infections. Humans with MV or VV codon 129 genotypes appear to be resistant (so far) to the BSE strain of prion (Brandel *et al.*, 2003), and inocula prepared from the brains of scapie-infected sheep target the challenged sheep differently according to PrP genotype (Goldmann *et al.*, 1994). For example, in the NPU Cheviot flock, although sheep of the ARQ/ARQ genotype appear to be resistant to scrapie following inoculation of the SSBP/1 scrapie strain (Goldmann *et al.*, 1994), and natural scrapie that circulates in the flock (Hunter *et al.*, 1996), they are susceptible to an experimental isolate of scrapie termed CH1641 and also to BSE (Foster *et al.*, 2001).

Studies in mice show that the incubation period of the disease depends not only on PrP genotype, but that the size and direction of this effect differ according to the prion strain (Bruce *et al.*, 1991; Carlson *et al.*, 1994). Pattison and Millson (1961) reported the occurrence of multiple scrapie strains over 30 years ago. Two experimentally passaged scrapie isolates produced dramatically different clinical signs in goats, labelled "nervous" or "drowsy", and syndromes were reproducible through subsequent i/c inoculations, implying that "true breeding" variants of the scrapie agent existed (Carlson, 1996). Over 20 phenotypically distinct TSE strains have been isolated in mice by serially passaging scrapie or BSE from a number of sheep, goat and cattle sources (Bruce, 2003). It has been suggested that each TSE strain represents a specific self-propagating PrP conformation (Safar *et al.*, 1998). Studies in mice indicate that TSE strains interact with genetic elements in the host and have a strong influence particularly on incubation period (and hence susceptibility to prion disease) and neuropathology. Experiments using panels of inbred mouse strains have shown that the BSE agent is indistinguishable from the agent causing vCJD, but differs from isolates of sCJD, reinforcing the idea that the vCJD epidemic in Britain is the result of consumption of contaminated beef products (Manolakou *et al.*, 2001). Incubation periods differ markedly for different TSE strains tested in the same mouse strain. Incubation periods are highly repeatable when equivalent doses of a specific TSE strain is administered to different groups of genetically uniform mice, but when different TSE strains are tested in the same mouse strain, the resulting incubation periods are clearly different (Bruce *et al.*, 1991). The incubation period is influenced largely by PrP genotype of the mouse and on the scrapie strain that is inoculated, with Prn-p^a showing the shortest incubation period for some TSE strains and Prn-p^b for other strains (Bruce, 2003). For example, when infected with the ME7

strain, Prn-p^a mice have a shorter incubation period than Prn-p^b mice. The reverse is true for the 22A strain whereby incubation times are shorter in Prn-p^b mice than in Prn-p^a animals. Incubation time, however, shortens through subsequent passage in mice expressing Prn-p^b (Bruce and Dickinson, 1987), reflecting perhaps host selection for more rapidly replicating prion mutants present in the 22A isolate (Carlson, 1996). For the ME7 and 22A strains, the incubation period in the F_1 heterozygote (Prn-p^{ab}) lies between those of the two parental phenotypes, and beyond the parental range, respectively (Bruce, 2003).

TSE strains differ in their ability to recognise and replicate in different neuronal populations in mice (Bruce, 1993), giving rise to characteristic "lesion profiles" (whereby strains are identified based on the distribution and severity of vacuolation in different areas of the brain and the presence or absence of amyloid plaques) (Bruce *et al.*, 1991; Fraser and Dickinson, 1968). Each combination of scrapie strain and mouse genotype has a characteristic lesion profile (Bruce and Fraser, 1991; Bruce *et al.*, 1991). The lesion profile stabilises on subsequent serial mouse-to-mouse transmissions; the same holds true for the incubation period, which also shortens after a few passages to produce a strain with characteristic properties (Bruce, 2003). However, it is possible that the major strain will be passaged together with minor variants. Hence, it is inappropriate to assume that the isolate contains only a single strain (Bruce, 1993) unless it is cloned (i.e. serially passaged several times at the minimum infective dose, to remove minor strains from the isolate) (Bruce, 2003). Whether or not the strains isolated in mice are representative of field strains in cases of natural scrapie is not known (Bruce and Fraser, 1991). However, since multiple strains have been isolated from most primary sources, this may be suggestive that mixed infections are common under natural conditions (Dickinson, 1976).

Although experimental TSE transmissions in mice have proved to be invaluable for our understanding of prion biology and disease pathogenesis, additional complexity arises because occurrence and development of disease depends not only on host genetics and agent characteristics, but also on the level of infectious challenge, route of exposure and age at exposure (Jeffrey *et al.*, 2001b). Furthermore, each combination of inbred mouse strains used in transmission studies contains only limited genetic variability compared with natural populations, and it is unlikely that all genes affecting the incubation period in natural populations are accounted for (Manolakou *et al.*, 2001).

Evidence from experimental infections in mice indicates that the incubation period is a function of the route of infection and the infective dose (Bruce *et al.*, 1991; McLean and Bostock, 2000). In general, the incubation period is lengthened when a smaller dose of agent is given or when a peripheral route of injection is employed instead of an i/c route. Experiments in mice have shown that the average incubation period increases linearly with logarithmic decrease in dose, and that the variability in incubation period increases linearly as the dose decreases (McLean and Bostock, 2000). Other authors have shown that the risk of infection depends not only on the total dose, but whether the dose was given as one or a series of challenges (Gravenor *et al.*, 2003). In hamsters infected orally with scrapie-infected brain homogenate, the attack rate was found to decrease with dilutions of homogenate, and subclinical infections were identified among the healthy survivors at 520 days post-infection (Baier *et al.*, 2003). An increase in the incidence of scrapie in sheep and a decline in the age at which the disease begins have been reported within affected flocks, and have been attributed to increasing levels of environmental contamination (Foster and Dickinson, 1989; Sigurdarson, 1991). In kuru, human females had shorter incubation periods compared to males, which may reflect a higher level of exposure among females rather than any difference in susceptibility (Huillard d'Aignaux *et al.*, 2002).

Hamir and others (2005) described the experimental transmission of scrapie to genetically susceptible Suffolk sheep by i/c and oral routes. The oral route resulted in longer incubation periods (956 ± 196 days) than i/c routes (570 ± 133 days). This is in agreement with findings from other studies involving the transmission of BSE to NPU Cheviot sheep via the same routes (Goldmann *et al.*, 1994). Although oral inoculation of PrP^{Sc} can lead to disease, this route is relatively inefficient when compared with direct inoculation of infectivity into the CNS (Tyler, 2004). Studies on the transmission of scrapie in hamsters suggest that oral inoculation was nearly a billion times less efficient in transmitting scrapie than was i/c inoculation and that the oral route of infection is associated with greater variability in incubation periods compared with parenteral ones (Prusiner *et al.*, 1985).

The survival of a proportion of sheep of susceptible genotypes following oral inoculation implies that factors other than PrP genotype may also govern the risk of infection by the oral route, and that uptake of infectivity may vary with factors such as rumen contents or maturity (Foster *et al.*, 2001). If the immune system of the gut plays a role in susceptibility to oral infection, it is possible that age will influence susceptibility to TSE infection since the amount of Peyer's patch (PP) tissue, part of the GALT (or number of lymphoid follicles in

these structures), decreases with age. Hence, the decreased amount of PP tissue may well contribute to reduced susceptibility to oral TSE infection in older individuals (Shmakov and Ghosh, 2001). Although oral exposure appears to be the most likely route of transmission for natural TSE infections, including vCJD, BSE and scrapie, the majority of published studies have carried out i/c inoculation of TSE-infected brain homogenates in experimental animals. In light of current epidemiological data, it appears that multiple oral-exposure events to BSE-contaminated beef and beef products that seemed likely in the UK, and the occurrence of vCJD mainly in young adults raise the possibility of age-related susceptibility or exposure to natural TSE infection (Head and Ironside, 2005).

1.1.6.3 Age of affected individuals

A striking feature of the vCJD epidemic has been the young age of most victims and the lack of any trend toward older ages in patients dying later in the epidemic (Figure 1.1A). The median age of onset of vCJD patients was 29 years three years ago (Will, 2003). Analyses, based on the assumptions that the incidence of vCJD is correlated with exposure to BSE-infected tissues, and that the incubation period distribution is unimodal for the PrP codon 129 methionine homozygous genotype (MM), show that the current age distribution of vCJD cases can only arise if younger individuals were either exposed to a greater extent to BSE through consumption of particular foodstuffs, were more susceptible to infection because of yet to be identified biological factors, or have shorter incubation periods (Ghani *et al.*, 1998). It is unlikely that the stable age distribution of cases arose from an age-dependent incubation period alone. This is because, under the latter hypothesis, we would expect an increasing age distribution over time, and models without age-dependent susceptibility or exposure do not provide good fits to the data (Ghani *et al.*, 2003). Furthermore, there is evidence that the incubation period is not linked to age, as has been found in kuru in humans (Huillard d'Aignaux *et al.*, 2002). Results demonstrate that individuals between 10 and 20 years of age are most susceptible to vCJD infection, with those over 40 years old being much less susceptible (Ghani *et al.*, 2003). However, it has been shown that differential dietary exposure alone to potential BSE- infected products does not decrease rapidly enough with age to reduce the risk of infection with vCJD in older adults, and that decreasing susceptibility with age is required to reproduce the characteristics of the age distribution of vCJD cases (Boëlle *et al.*, 2004).

Although some parallels can be drawn with other human TSEs such as kuru, direct comparison cannot be made since oral transmission and the species barrier (which is absent

for kuru) have been shown to lengthen and increase variability in the incubation period in animal experiments (Diringer *et al.*, 1998; Kimberlin and Walker, 1978). The occurrence and development of acquired TSEs like vCJD is complex, depending not only upon host genetics, but also on the level of exposure to the infectious agent, age at exposure and the species barrier. Hence, it is worth emphasising that extrapolations to acquired human TSEs need to carefully consider all of these limitations. Although recent data raise the possibility that the epidemic has peaked (Andrews *et al.*, 2003), models rely on a range of assumptions including important determinants like the incubation period of BSE in humans or the infectious dose of BSE for humans, and there remains uncertainty about the likely size of the total vCJD epidemic.

For BSE, there is an apparent age-dependent risk of infection with calves having a higher risk of infection than adult cattle (Arnold and Wilesmith, 2004). Cases of BSE typically peak in cattle at 5-7 years (Defra, 2004) (Figure 1.1B). The majority of clinical cases of scrapie occur in sheep between 2 and 4 years of age (Baylis *et al.*, 2004; Redman *et al.*, 2002) (Figure 1.1C). However, cases have been reported in sheep as young as 7 months (Sigurdarson, 1991) and in animals as old as 11 years (Parry, 1962). In captive cervids, most cases of CWD occur in animals 2–3 years of age (Figure 1.1D); however, the disease has been reported in cervids as young as 17 months and as old as >15 years of age (Williams *et al.*, 2002). Why the peak incidence is in young adults must be a matter of conjecture in the present state of knowledge. Nonetheless, the age distribution of scrapie is consistent with the hypothesis that most victims probably encounter the pathogen either prenatally or in early post-natal life and that the long incubation period of the disease results in a peak incidence at 2 or 3 years old (Dickinson *et al.*, 1964). As mentioned previously, some studies indicate that in field outbreaks of scrapie (Hunter *et al.*, 1997) or following oral dosing (O'Rourke *et al.*, 1997), between 12 and 20% of susceptible genotypes do not develop the disease. Other authors have shown that naturally infected Suffolk sheep flocks do not demonstrate 100% scrapie disease in susceptible genotypes (Jeffrey *et al.*, 2001b). This implies that other factors may be involved in determining susceptibility to the disease. Diaz et al. (2005) have shown that animals first exposed to infection at older ages seemed to be less susceptible to scrapie than animals encountering infection for the first time early in life. Although this age effect may be explained by a higher susceptibility of younger animals to scrapie infection (Matthews *et al.*, 2001), a relationship between age at infection and age at which symptoms appear also exists (Woolhouse *et al.*, 1998). Therefore, animals exposed at older ages may have been culled or died for other reasons, resulting in insufficient time to develop scrapie

symptoms, although available data suggest that the average incubation period for all susceptible genotypes is unlikely to be significantly greater than 2 years (Woolhouse *et al.*, 1998). Another complication arises in understanding how the age-incidence curve for scrapie interacts with the background survivorship (or the natural mortality of sheep). This is reflected in one study (Chase-Topping *et al.*, 2005), which found that the peak in scrapie deaths approximates the point at which 50% of the susceptible animals in the flock, in which scrapie was not diagnosed, were being removed. Whether these removals were the result of susceptible genotypes being in poorer condition and consequently removed at younger ages, or whether they developed pre-clinical scrapie is not known.

Due to the age range considered and the route of transmission, biological factors involved in the maturation of the immune system of the gut and peaking in adolescent years may promote prion replication and consequent development of disease. Previous studies suggest that the incidence of scrapie infection decreases with the age at which animals are exposed and infection is thought to occur in young sheep less than 9 months of age and (Hourrigan *et al.*, 1979). During this period of high susceptibility to infection, the ileal PP is the major GALT possessing an extensive bed of FDCs and specialized epithelium (follicle-associated epithelium or FAE) actively engaged in the transport of antigens and macromolecules from the gut to the underlying lymphoid tissue (Press *et al.*, 2004). In humans, the number of PPs was found to be higher in the small intestine of teenagers than in young children and adults (Cornes, 1965). The same holds true for sheep (Reynolds and Morris, 1983) and cattle (Carlens, 1928) whereby the amount of PP tissue decreases with age, with a peak occurring in adolescent years. Assuming that particle uptake capacity is a function of the area occupied by FAE overlying PPs, these differences may explain susceptibility of young people and lambs to vCJD and scrapie, respectively (Shmakov and Ghosh, 2001). There is some variation between different individuals (sheep, cattle and humans) in all age groups (Carlens, 1928; Cornes, 1965; Reynolds and Morris, 1983), and this variation may well contribute to individual susceptibility to prion diseases.

In experimental studies of mice, the age at time of peripheral injection with scrapie is known to influence the length of the incubation period and susceptibility to scrapie (Outram *et al.*, 1973). I/p injections of ME7 scrapie were found to be 100% lethal in young adult mice and killed only about 80% of neonatal mice, which also showed significantly prolonged incubation periods (Outram *et al.*, 1973). Paradoxically, a proportion of the neonatal mice injected subcutaneously with ME7 developed scrapie after shorter incubation periods

compared to weanlings, and if neonatal mice were challenged with higher scrapie doses, the proportion of mice with shorter incubation periods increased. Survivors only occurred in the newborn group given the lowest titre (Outram *et al.*, 1973). Based on these findings, it was proposed that neonatal mice that survive peripheral scrapie challenge and those with prolonged incubation periods have an impaired ability to replicate scrapie and may lack a mature cell population that could be undergoing differentiation after birth and required for the initiation of scrapie pathogenesis (Outram *et al.*, 1973). Hence, the outcome would ultimately depend on the balance of an individual's developmental state and the dose of agent used (Outram *et al.*, 1973). Other work has shown neonatal mice to be very susceptible to agent injected by various s/c routes (Ierna, 2001; Outram, 1976). After s/c challenge of neonatal severe combined immunodeficient (SCID) and CB20 mice with the ME7 strain, a very high proportion developed scrapie with short incubation periods (Ierna, 2001). SCID mice inoculated at 14 days or older did not develop scrapie with short incubation periods and were relatively resistant to infection. These results imply that neonatal mice may possess an efficient mechanism by which infectivity can quickly gain access to the CNS following s/c challenge with scrapie. Since similar proportions of neonatally challenged SCID and CB20 mice succumbed to scrapie after short incubation periods, similar pathogenic mechanisms (following s/c inoculation) may be involved and may not necessarily be FDC-dependent (Ierna, 2001).

Although studies in mice demonstrate an age-effect, direct comparisons cannot be made to other species because of differences in development of the lymphoreticular system (LRS) in mice and the route of inoculation used in these experiments. Humans, sheep and cattle for example, are more immunologically mature at birth than mice. In mice, the lymphoreticular system and in particular FDCs are still undergoing development during the first two postnatal weeks (van den Berg and Dijkistra, 1995) and therefore, may be unable to replicate the scrapie agent. Splenic FDCs do not become fully mature until about 2-4 weeks after birth and the time at which FDCs mature coincides with the normalisation of the scrapie incubation period to that of the adult (Ierna, 2001). Furthermore, unlike i/p infection, oral exposure to the scrapie agent appears to be less or not dependent on the spleen (Kimberlin and Walker, 1989a). This has been suggested by Kimberlin and Walker (Kimberlin and Walker, 1989a; Kimberlin and Walker, 1989b) who observed that splenectomy does not prolong the incubation period after intragastric infection as opposed to the prolongation observed following infection via the i/p route.

So far, there is no clear biological explanation for the apparent age distribution of TSEs. Although a relation with puberty may be hypothesized, evidence should be sought to support such a possibility since the age-susceptibility relationship is crucial in determining whether or not the age of vCJD cases is increasing and, if not, whether it should be expected to do so.

1.1.7 Pathogenesis of the TSEs

Oral exposure appears to be the most likely route of transmission for natural TSE infections including natural scrapie, BSE, CWD, TME, Kuru and vCJD (Gajdusek, 1977; Hadlow *et al.*, 1982; Marsh and Bessen, 1993; Shmakov and Ghosh, 2001; Williams and Young, 1980). For example, it is believed that kuru was spread through cannibalism, which was associated with ritual mourning among the Fore people in New Guinea (Gajdusek, 1977). A more recent example was the emergence of a number of cases of vCJD in 1996, which suggested that these cases might be causally linked to the BSE epidemic in UK cattle (Will *et al.*, 1996) and was almost certainly due to the consumption of BSE-infected meat products (Bruce *et al.*, 1997). In sheep, other potential sources of natural infection which have been shown to be effective experimentally, are scarification (Taylor *et al.*, 1996) and horizontal transmission (Andréoletti *et al.*, 2002a) by consumption of foetal membranes from scrapie-infected sheep (Pattison *et al.*, 1972). Several studies have been carried out on the transmission of scrapie in sheep by embryo transfer. In one study, scrapie was found not to be transmitted to offspring via the embryo nor was the infective agent transmitted to recipient ewes during embryo transfer procedures (Foote *et al.*, 1993). Another study showed that scrapie was transmitted via embryo transfer (Foster *et al.*, 1996). These experiments have revealed conflicting results and do not provide an adequate basis to confirm that embryo transfer is a truly safe method of preventing the transmission of scrapie (Foote *et al.*, 1993; Foster *et al.*, 1996; Foster *et al.*, 1992). Further work is required to provide a definitive answer to embryo transfer and scrapie transmission. Work to date argues against the transmission of scrapie *in utero*. No infectivity was detected in foetal tissues from naturally infected dams (Hadlow *et al.*, 1982; Hourrigan *et al.*, 1979) and no apparent PrP^{Sc} was detected within the foetus (Andréoletti *et al.*, 2002b). Epidemiological evidence suggests that if vertical transmission occurs at all, it does not account for the majority of cases in heavily infected flocks (Elsen *et al.*, 1999; Woolhouse *et al.*, 1998).

Many studies have used experimental mice and hamster scrapie models in an attempt to elucidate the pathogenesis of TSEs. It is important to note that several factors determine the course of TSE infection including the infecting TSE strain, the host PrP genotype and the

route and dose of infection (Farquhar *et al.*, 1994). For example, comparatively different ranges of incubation period have been obtained using many mouse scrapie models, which vary mainly in agent strain and *Sinc* genotype of the mouse (Kimberlin and Walker, 1988). Although the i/c route of infection is used extensively for bioassays of infectivity, it does not represent a clinically relevant model for scrapie. Eklund *et al.* (1967) performed a major study on "Chandler" scrapie in which the agent was injected s/c. It was established that extraneural events of scrapie pathogenesis (which were bypassed by the i/c route) were involved after peripheral challenge. This finding was confirmed by Hadlow *et al.* (1982) who showed that after peripheral exposure, pathogenesis necessarily involves the LRS before the CNS. The importance of the immune system in the pathogenesis of TSEs is further exemplified by alteration of the immune cell repertoire, either by splenectomy (Fraser *et al.*, 1996; Kimberlin and Walker, 1989a), disease (Lasmézas *et al.*, 1996) or genetic manipulation (Brandner *et al.*, 2000), which can alter the progression of prion disease. Since oral exposure has been incriminated as one of the most probable routes of transmission leading ultimately to neuroinvasion, it seems appropriate to investigate this route of infection and to identify potential target cells of scrapie infectivity for purposes of prophylaxis and therapy.

1.1.7.1 The role of GALT in oral TSE Pathogenesis

The appearance of vCJD and evidence suggesting that this new human prion disease resulted from interspecies transmission of BSE (Bruce *et al.*, 1997) has renewed scientific interest in the pathogenesis of oral transmission of TSEs, as this represents the most obvious route of human exposure to BSE-infected material.

Although lesions are restricted to the CNS, the pathogenesis of infection implies an initial replicative phase of the infectious agent in the lymphoid tissues prior to invasion of the CNS and development of clinical disease (Hadlow *et al.*, 1982; Sigurdson *et al.*, 1999). The lymphoid system is essential for transport of prions from the periphery to the CNS as evidenced by the impairment of CNS disease in immune deficient mice (like SCID, $RAG^{-/-}$ or μMT mice) after i/p inoculation, and development of CNS disease after i/c inoculation (Fraser *et al.*, 1996; Klein *et al.*, 1997; Lasmézas *et al.*, 1996). When rodents were orally exposed to scrapie, PrP^{Sc} accumulation occurred first in PPs and ganglia of the enteric nervous system (Beekes and McBride, 2000) long before detection in the CNS (Farquhar *et al.*, 1994). PrP^{Sc} was also first detected in lymphoid tissues (including the retropharyngeal lymph node, tonsil, PPs and ileocaecal lymph node) following experimental oral infection of

mule deer fawns with CWD (Sigurdson *et al.*, 1999). In one study (Kimberlin and Walker, 1989a) it has been shown that mice infected intragastrically with scrapie had detectable infectivity in PPs and cervical lymph nodes as early as one week post-inoculation. Other authors (Wells *et al.*, 1994) have shown that BSE can be transmitted orally to cattle with infectivity detectable in the ileum of calves at 26 weeks post-inoculation. Kuru, Creutzfeldt-Jakob disease and scrapie have been transmitted to squirrel monkeys (*Saimiri sciureus*) after oral exposure to infectious tissue (Gibbs *et al.*, 1980). These findings support oral exposure as a natural route of infection for TSEs.

1.1.7.1.1 PPs: What makes these structures so interesting?

PPs are collections of lymphoid follicles, located along the anti-mesenteric border throughout the small intestine, and present in a wide variety of animals including birds, rodents, ruminants and man (Griebel and Hein, 1996; Poskitt *et al.*, 1984) (see Figure 1.2). Historically, butchers expressed concern (more so than physicians) over PPs which appeared as thimble-shaped structures on the surface of intestines and as casings for sausages, making them less marketable (Carlson and Owen, 1987).

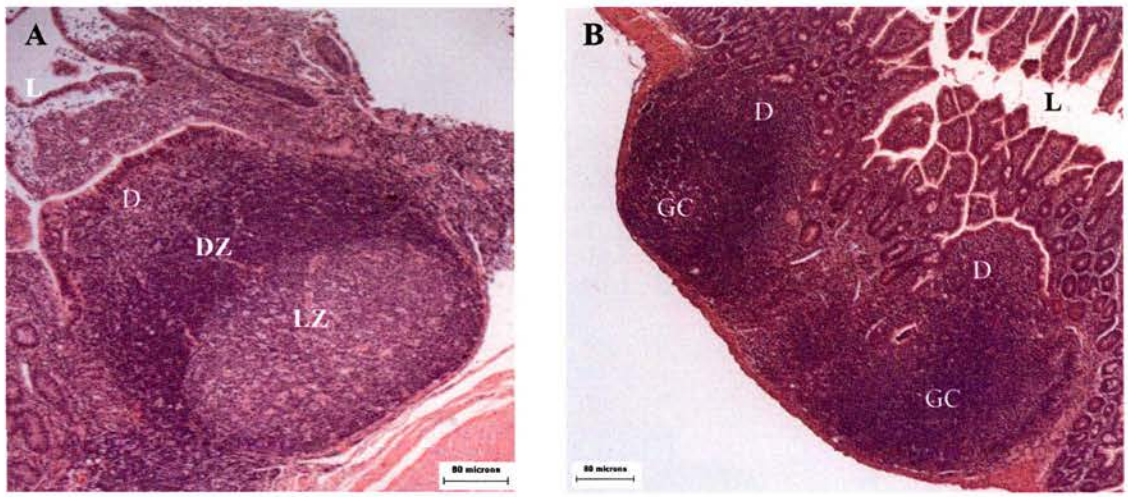

Figure 1.2 PP structures in sheep and mice. **A.** Lymphoid follicle of the ileal PP in a 6 year-old Cheviot sheep **B.** PP in a 35 day-old C57 BL mouse containing two germinal centres. LZ=light zone of lymphoid follicle; DZ=dark zone of lymphoid follicle; D=dome; L=lumen; GC=germinal centre.

PPs of the small intestine were first described in 1677 by a Swiss physician, Johann Conrad Peyer (Griebel and Hein, 1996), although patches may actually have been discovered by Marco Aurelio Severino in 1645 who may not have proclaimed his findings sooner (Heel *et al.*, 1997). These organs form a major part of the GALT, which plays a critical role in the

induction and dissemination of immune responses at mucosal surfaces (Griebel and Hein, 1996), and which also consists of colonic and caecal patches, appendix and isolated lymphoid follicles throughout the intestine from the oesophagus to the rectum (MacDonald and Spencer, 1994).

PPs occur with increased frequency in the ileum, especially the terminal ileum (Cornes, 1965; van Kruiningen *et al.*, 2002). It has been theorised that intestinal contents progress at a relatively slower pace near the ileocaecal junction thus resulting in increased contact time between antigens and mucosa and consequently, leading to the development of PPs in response to this increased contact (Carlson and Owen, 1987).

The amount of PP tissue in the distal ileum bears clinical relevance. With respect to vCJD, the quantity of lymphoid tissue encountered during some clinical procedures for example, lower gastrointestinal endoscopy, may contribute to the risk of transmitting PrP to the next patient. Axon *et al.* (2001) have indicated that the greatest risk arises from biopsy of the terminal ileum, where PPs are highly concentrated and may contain significant levels of PrP for a patient incubating vCJD. Because these lymphoid organs are undoubtedly an inherent part of natural TSE pathogenesis, it is important to examine briefly the comparative aspects of the structure and function of PPs in the different species. Furthermore, the variation in PP number, size and area in different individuals is greater than is often appreciated and whether this variation is related to susceptibility to TSE infection raises an intriguing possibility.

1.1.7.1.2 Heterogeneity of PPs in the different species

Two distinct groupings are observed with respect to the anatomical and developmental patterns of the amount of PP tissue found in various regions of the intestine. In ruminants, pigs, horses, dogs and humans (group I species) the PP tissue is unequally distributed along the small intestine with 80% to 90% of the total mass of PP tissue occurring in the ileum (HogenEsch *et al.*, 1987; Pabst *et al.*, 1988; Reynolds and Morris, 1983). In sheep, the total weight of PP tissue is greater than any other single lymphoid tissue by around 6 weeks after birth, accounting for about 1.2% of the body weight (Reynolds and Morris, 1983). The ileal PP forms a single continuous aggregation of lymphoid follicles that can extend up to 2.5 m (in 6 week-old lambs) from the ileocaecal junction, proximally, accounting for about 90% of the total mass of PP tissue (Reynolds and Morris, 1983; Reynolds and Pabst, 1984). The ileal PP in a lamb contains about 100,000 follicles (Reynolds, 1987). In young pigs, the ileal PP may extend up to 2 m in length (Pabst *et al.*, 1988). In humans, PPs are distributed in small clusters throughout the gastrointestinal tract, and as in sheep, are most concentrated in the

terminal ileum (Cornes, 1965; van Kruiningen *et al.*, 2002). The number of follicles per patch vary from as few as 5 to several hundred (Cornes, 1965)). Although a similar bias seems to exists for horses, the amount of PP tissue occurring in the ileum has not been accurately measured (Carlens, 1928). A continuous ileal PP is also found in young carnivores, such as blue foxes and mink (Landsverk *et al.*, 1991).

In rabbits, rodents and chickens (Group II species), PPs occur at random intervals along the jejunum and ileum (Abe and Ito, 1978; Befus *et al.*, 1980; Waksman, 1973). In rats, there are approximately 18 to 26 PPs, the number of macroscopically visible PPs and their sizes increasing with age in young rats up to 6 weeks postpartum (Hummel, 1935). The number of follicles in each patch varies from as few as 4 to 6 to about 20 in the largest patch near the ileocaecal junction. Mice have approximately 9 PPs in the small intestine (Mayrhofer, 1984). In rabbits, PP number ranges from 2 to 10 per animal, consisting of approximately 40 to 50 follicles (Faulk *et al.*, 1970). In rabbits and chickens, specialized GALTs are also present in other areas of the small intestine. These include the sacculus rotundus and an exaggerated appendix in rabbits, and the bursa of Fabricius in chickens (Griebel and Hein, 1996). Histologically, the rabbit appendix and the bursa of Fabricius resemble the sheep ileal PP, while the PPs in the rabbit resemble the sheep jejunal PPs (Reynolds *et al.*, 1985).

The PPs develop differently between species of these two groups. In Group I, PPs develop well before birth in the ileum (by 110 days gestation in lambs) and jejunum (by 75 days gestation in lambs) (Reynolds *et al.*, 1985). The only other lymphoid organs which are histologically mature before birth are the thymus and avian bursa of Fabricius (Archer *et al.*, 1963; Cooper and Lawton, 1973). Since external antigen is not required for the development of jejunal and ileal PPs, the follicles in PPs are not comparable to germinal centres (Reynolds and Morris, 1983). The jejunal and ileal PPs, however, develop differently after birth. The ileal PPs reach their maximum size early in life and then involute in a manner similar to that of the thymus. For example, in sheep, the ileal PP begins to involute from about 12 weeks after birth with only a few follicles remaining in the ileum by 18 months of age (Reynolds and Morris, 1983). In humans, the number of PPs increases to a maximum in the entire small intestine by late adolescence and then decreases with increasing age (Cornes, 1965). Van Kruiningen *et al.* (2002) studied the distal ileum of humans and concluded that a greater amount of lymphoid tissue was present in 21 to 30 year-olds than in older age groups, and that the area of PP tissue declines progressively with age. However, the study included few young subjects (none within the age category, 0-15 years) and different criteria were

used (from Cornes, 1965) in defining a PP. The ileal PP in minks and blue foxes is thought to regress early, probably at about one year of age (Landsverk *et al.*, 1991). The jejunal PPs, by contrast, persist throughout the life of the animal, although the density of follicles may decrease with increasing age (Griebel and Hein, 1996).

In Group II species, PPs develop post-natally, usually in the first 2-4 weeks after birth (Abe and Ito, 1978; Befus *et al.*, 1980; Sminia *et al.*, 1983; Waksman, 1973) and, as for the jejunal PPs of Group I species, also persist into old age (Griebel and Hein, 1996). The development of PPs in Group II species depends on antigenic stimulation since they remain small and poorly developed in germ-free mice.

1.1.7.1.3 Function of PPs

The two types of sheep PPs (ileal and jejunal) can be distinguished by their microanatomy and cellular composition of follicles (Hein *et al.*, 1989; Larsen and Landsverk, 1986; Reynolds *et al.*, 1985). The ileal PP of sheep consists of densely packed, long, sac-like lymphoid follicles, each separated by a connective tissue sheath. The follicles contain mostly B cells (95% $sIgM^+$) and few T cells (<0.5% $CD4^+$). Jejunal PPs on the other hand, have small, pear-shaped follicles separated by large interfollicular areas, and contain up to 15% T cells (Griebel and Hein, 1996). Studies investigating the functions of PPs have been performed mostly on rodents and based on interpretation of the data obtained, various authors have cast PPs as secondary lymphoid organs (where mature lymphocytes become stimulated to respond to invading pathogens). In rats, PPs were recognised as a site of generation of antigen-specific IgA precursors, which then migrate to other regions of the gut lamina propria (Husband *et al.*, 1977). In another study, rabbit PPs were found to contain an enriched population of IgA precursors that seeded into other areas of the gut (Craig and Cebra, 1971). Microfold cells (or M cells), which have been described (Nicoletti, 2000), present a possible route for transport of macromolecules and micro-organisms from the gut lumen to the underlying mucosal tissue. The concept of PPs as secondary lymphoid organs contains the element that antigen uptake (including prion uptake) occurs in these specialized cells or M cells (Heppner *et al.*, 2001; Nicoletti, 2000). Reynolds (1987) conducted a series of experiments in sheep, which ultimately classified sheep ileal PPs as primary lymphoid organs (or organs where immune cells develop and mature to the stage at which they are able to respond to a pathogen). In sheep, the ileal PP is the most significant source of B cells for export to other tissues (unlike laboratory rodents where the major source is the bone marrow), and antigen does not have to be present for lymphopoiesis to occur (Reynolds, 1987). Removal of the ileal PP in sheep foetuses near to term or shortly after birth resulted in

lambs remaining B-cell deficient for at least one year and failing to produce antibodies (Gerber *et al.*, 1986). Most PPs in sheep have stopped producing B cells by the time the animal is fully grown (Reynolds, 1987). Other more recent studies have also supported the role of the ileal PP as a primary lymphoid organ (Reynaud *et al.*, 1995; Reynaud *et al.*, 1991).

1.1.7.1.4 Role of PPs in TSE Pathogenesis

PrP^{Sc} has been widely accepted as a marker of TSE infectivity and used to investigate the pathogenesis of the TSE agent. Following peripheral inoculation, abnormal PrP usually accumulates in a variety of lymphoid tissues well before the CNS is involved (Sigurdson *et al.*, 1999; Williams and Miller, 2002). PPs show early presence of infectivity (Kimberlin and Walker, 1989a) and PrP^{Sc} (Beekes and McBride, 2000) after oral exposure to the TSE infectious material, and are the most probable sites for the intestinal uptake of prions (Shmakov and Ghosh, 2001) (Figure 1.3). Andréoletti *et al.*, (2000) have suggested that natural scrapie infection occurs via the oral route and that the ileal PP acts as a likely primary entry site of the scrapie agent. In one study (Terry *et al.*, 2003), PrP^{Sc} staining was demonstrated in the follicles of PPs of the distal ileum of cattle orally exposed to the BSE agent. This finding supports the suggestion that, in common with some other models of TSEs, the distal ileum is a probable route of entry of the agent. However, in the same study, no immunostaining was detected in the lymphoid tissue of the distal ileum of naturally occurring clinical cases of BSE; this finding has been attributed to the possibility of natural field cases of BSE having been exposed to lower doses of the agent or the inability to detect decreased infectivity after an incubation period of 5 years (Terry *et al.*, 2003). It is likely, therefore, that the importance of lymphoreticular amplification varies with the dose and possibly the strain of the infecting agent. PrP^{Sc} staining has also been reported in PPs and tonsils in non-human primates after natural and experimental infection by the BSE agent (Bons *et al.*, 1999). Additional evidence supporting a key role for PPs in oral infection with the TSE agent comes from the fact that mice with genetic defects resulting in decreased numbers of PPs are resistant to oral challenge with prions (Prinz *et al.*, 2003).

Following oral exposure of hamsters to scrapie, pathological PrP accumulations were detected in PPs within cells of the FAE which have M cell morphology (Beekes and McBride, 2000). M cells are specialized epithelial cells of the FAE and occur only over mucosal lymphoid follicles (Kucharzik *et al.*, 2000). They are the key sites of antigen sampling in the gut mucosa and play a central role in the initiation of gut immune responses

by transporting enteric pathogens from the gut lumen to the underlying lymphoid tissue (Nicoletti, 2000). Experiments using an *in vitro* cell culture model suggest that M cells have the ability to transcytose the TSE agent across the gut epithelium (Heppner *et al.*, 2001). Oral inoculation with prions in mice deficient in enteric lymphocytes but with preserved M cells indicates that it is the M cells and not the enteric lymphocytes that are critical in initial entry of prions (Prinz *et al.*, 2003).

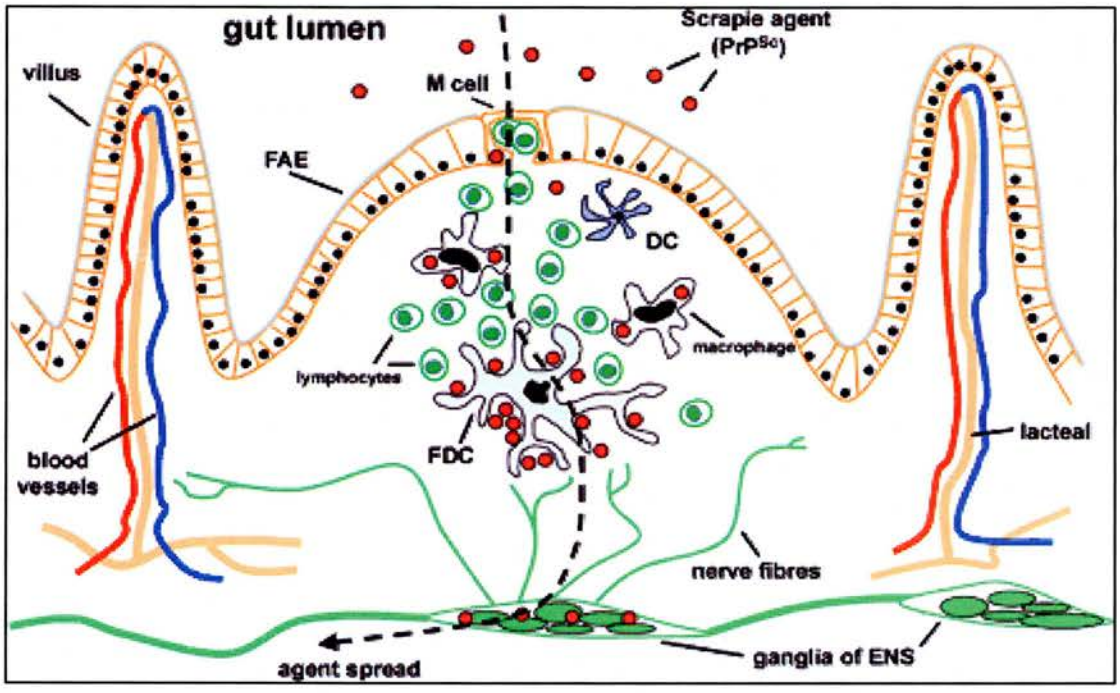

Figure 1.3 Possible spread of scrapie infectivity from the gut lumen to the nervous system following oral infection (route indicated by dotted line). Soon after ingestion, PrP^{Sc} is detected readily within Peyer's patches upon FDCs, within macrophages, within cells with morphology consistent with that of M cells and within ganglia of the enteric nervous system (ENS). These observations suggest that following uptake of scrapie infectivity from the gut lumen infectivity accumulates upon FDCs in Peyer's patches and subsequently spreads via the ENS to the CNS. DC=Dendritic cell; FAE=follicle-associated epithelium (taken from Mabbott and Bruce, 2001).

It has also been suggested that intestinal mucosal epithelial cells may provide a route for entry of PrP^{Sc}. This is based on immunoreactivity found in intestinal cells of the small and large intestine and a laminin receptor found on human small intestinal mucosa which can bind PrP^{Sc} (Rieger *et al.*, 1997; Shmakov *et al.*, 2000). It is important to note that M cells differ in their ability to "sample" specific pathogens depending on species and location in the gut. For example, the FAE overlying the ileal PP in ruminants does not constitute a population of typical M cells, but instead comprises an homogenous population of epithelial cells capable of phagocytosis (Landsverk, 1988; Landsverk *et al.*, 1987). Furthermore, calf

M cells in the FAE of jejunal PPs, which are comparable to those of mice in structure (Landsverk *et al.*, 1987), do not appear to take up latex or parapox virus (Landsverk, 1988). In lambs, the FAE of the ileal PP, but not of the jejunal PP, secretes small membrane-bound carbonic-anhydrase-positive particles, which move into the domes and central areas of underlying follicles (Landsverk *et al.*, 1987; Landsverk *et al.*, 1990). Despite these differences, the FAE overlying calf and lamb ileal PPs, as well as jejunal PPs, has a well-developed mechanism for the transcytosis of luminal material (Landsverk *et al.*, 1991), and does not preclude the possibility that M cells (and homogenous epithelial cells in the ileum of ruminants) are the portal of entry for prions. Whether these regional and species differences apply to uptake of prions requires direct investigation.

In PPs, dendritic cells form a dense layer of cells in the subepithelial dome just beneath the FAE and in close contact with M cells (Kelsall and Strober, 1996). Migratory bone marrow-derived dendritic cells are centrally involved in transport of proteins both within PPs and to mesenteric lymph nodes (Banchereau *et al.*, 2000; Huang *et al.*, 1996). It is probable that in deer (which possibly demonstrate an immunobiology similar to that in sheep), initial uptake and propagation of PrP^{Res} could occur in the ileal PPs and tonsils and within dendritic cells that act as a cellular bridge, transporting PrP^{Sc} from the gut lumen via the lymphatics to the lymphoid tissue (Huang *et al.*, 1996) and emigrating via the lymphatic system to the ileocaecal and retropharyngeal lymph nodes (Sigurdson *et al.*, 1999).

1.1.7.2 The origin and characteristics of FDCs

FDCs are specialized antigen-trapping cells that have only been detected in the lymphoid tissue, located within B-cell follicles (Humphrey *et al.*, 1984; Radoux *et al.*, 1984). They have morphological similarities or critical markers in common with several other cell types including reticular cells, pericytes, some antigen-transporting cells, mononuclear cells and endothelial cells, and this has prompted the suggestion that each of these cell types may represent an FDC precursor and may explain the origin of FDCs (Tew *et al.*, 1982). However, the origin of this unique cell type has not been unequivocally established. FDCs may be derived from primary lymphoid tissue and need not develop from local stromal cells originating in the local secondary lymphoid tissue (Kapasi *et al.*, 1998; Tew *et al.*, 1982). They can be distinguished from other immune system cells based on their cytoplasmic extensions (dendrites), which form a dense network in the germinal centre and make contact with germinal centre B-cells (MacLennan, 1994). Their location is restricted to the light zones of germinal centres of the lymphoid follicles and FDCs have the ability to trap and

retain immune complexes on their surfaces for prolonged periods of time (from months to years) through the presence of complement receptors (CR1, CR2 and CR3) and the F_c receptor (Fang *et al.*, 1998; Liu *et al.*, 1997; Schriever *et al.*, 1989; Sellheyer *et al.*, 1989; Tew *et al.*, 1990; Tew *et al.*, 1997). FDCs should not be confused with the typical dendritic-type cells, which may be found in virtually any area of the body and may be thought of as sentinels of the immune system (Ibrahim *et al.*, 1995). Dendritic-type cells, rather than presenting antigens to B-cells, interact with, and process and present antigens to T-cells in a highly efficient fashion (Cella *et al.*, 1997; Schuler *et al.*, 1997; Shortman *et al.*, 1997; Steinman, 1996).

FDCs are resistant to high doses of radiation and appear to be very long-lived cells with a slow turnover, for example, antigen-bearing FDCs are present in murine hosts for more than a year (Tew *et al.*, 1990; Tew *et al.*, 1982). According to Tew and colleagues (Tew *et al.*, 1982), this inevitably presents a problem in FDC pathogenesis studies since radiation does not eliminate FDCs from the system, and host FDCs are likely to be present following reconstitution with donor bone marrow. Consequently, difficulties may arise when trying to detect FDCs of donor origin.

1.1.7.2.1 FDCs and their role in TSE pathogenesis

It has been suggested that the host immune system may act as a critical susceptibility factor (Partanen, 2003) particularly as PrP^{Sc} can first be detected in lymphoid tissues on FDCs (Mabbott and Bruce, 2001). Mature FDCs express high levels of the host PrP (PrP^{C}), which may be converted to PrP^{Sc} (Burthem and Roberts, 2003), and is essential for TSE replication in lymphoid tissues (Brown *et al.*, 1999). PrP^{Sc} usually accumulates on FDCs of lymphoid tissues of patients with vCJD (Hill *et al.*, 1999), sheep naturally infected with scrapie (van Keulen *et al.*, 1996) and rodents inoculated with scrapie via the peripheral route (Brown *et al.*, 1999; Mabbott *et al.*, 2000; McBride *et al.*, 1992) and appears to be critical for neuroinvasion. The importance of FDCs in TSE pathogenesis has been demonstrated through several studies. Mabbott *et al.* (2000) have shown that treatment of mice with lymphotoxin β-receptor and human immunoglobulin (LTβR-Ig) before or shortly after peripheral scrapie challenge with the ME7 scrapie strain, significantly extended incubation periods, compared to that of control mice treated with human immunoglobulin. FDCs depend on expression of lymphotoxin α/β receptor by B-cells (Endres *et al.*, 1999), and blocking of this signalling with LTβR-Ig is believed to result in a loss of mature FDCs and consequently, a substantial slowing of the disease. However, after i/c challenge, treatment with LTβR-Ig had no effect

on disease development and scrapie pathogenesis in the brain (Mabbott *et al.*, 2000). FDCs can, therefore, serve as potential targets for therapy in peripherally transmitted TSE diseases like vCJD. It is worth noting that the authors inoculated mice intraperitoneally and intracerebrally. These routes are however, not equivalent to natural routes of oral infection, and the importance of FDCs as an amplifying site for prion infectivity after oral infection remains unsettled.

1.1.7.2.2 The use of immunodeficient and chimeric mice in determining the role of FDCs in TSE pathogenesis

The role of FDCs in TSE pathogenesis has been investigated using SCID mice. SCID mice lack mature FDCs (Kapasi *et al.*, 1993) and are easily transplanted with a variety of donor cells. Hence, they provide a useful tool for assessing the role of FDCs in TSE pathogenesis. In SCID mice, the lack of FDCs is demonstrated by the absence of antigen localization on FDCs in lymph node and splenic follicles of passively immunized mice, and by the absence of labelling with the monoclonal antibody FDC-M1 (Kapasi *et al.*, 1993). Kapasi and colleagues (1993) have shown that FDC networks develop rapidly in SCID mice reconstituted with B- and T-cells; this indicates that SCID mice have SCID precursors, and that immature FDCs can be induced to mature by bone marrow grafts from immunocompetent donors.

In mouse scrapie models, mature FDCs express high levels of the host PrP (PrP^C), which is essential for TSE replication in lymphoid tissues (Brown *et al.*, 1999). Studies using SCID mice have indicated involvement of FDCs in scrapie pathogenesis of the ME7 scrapie strain. Brown *et al.* (1999) used PrP chimeric mice, by γ-irradiating $PrP^{+/+}$ or $PrP^{-/-}$ mice (which destroys lymphocytes, myeloid cells and their progenitors, but not FDCs), and then grafting bone marrow from PrP-deficient ($PrP^{-/-}$) knockout mice into PrP-expressing ($PrP^{+/+}$) mice and vice versa. It was found that replication of the mouse-passaged ME7 scrapie strain in spleen involves FDCs with no evidence for any direct involvement of circulating B-cells or other bone marrow-derived cells. Replication of the scrapie agent did not occur in mice with PrP-deficient FDCs, even when bone marrow was grafted from PrP-expressing mice, that is, PrP^C on FDCs was seen only when the recipient expressed a functional PrP gene. PrP status of bone marrow-derived cells did not influence the levels of infectivity measured in the spleen, nor did it affect the time of development of neurological disease in mice infected intraperitoneally.

Tumour necrosis factor-α (TNF-α) signalling from B-lymphocytes is important for the maturation of FDCs (Mabbott and Bruce, 2001). TNF-receptor 1 (TNF-R1) is expressed on the FDC and/or its precursor (Tkachuk *et al.*, 1998). Signalling is mediated via this receptor, and mice deficient in either TNF-α or TNF-R1 lack mature FDCs (Le Hir *et al.*, 1996; Pasparakis *et al.*, 1996). After peripheral challenge, TNF-α knockout mice either fail to develop scrapie, or few develop clinical disease after extended incubation periods (Brown *et al.*, 1999).

1.1.7.2.3 The Rocky Mountain laboratory (RML) scrapie isolate

There are several laboratory mouse-passaged TSE strains that have distinct incubation periods and neuropathological characteristics; findings suggest that some strains may target different cell populations in peripheral tissues. For example, studies (Blättler *et al.*, 1997; Klein *et al.*, 1997) using the Rocky Mountain Laboratory (RML) scrapie isolate have given remarkably different results from that obtained by Brown *et al.* (1999). Blättler *et al.* (1997) found that replication of the scrapie agent took place in the LRS and could be restored by PrP-expressing lymphocytes and myeloid cells although the specific lymphohaemopoietic stem cells or LSC-derived cells involved in prion replication have not been determined (Raeber *et al.*, 1999). Several authors concluded that mature B-lymphocytes are critical for neuroinvasion by scrapie after peripheral exposure, and discounted the crucial role of FDCs in TSE pathogenesis (Klein *et al.*, 1998; Raeber *et al.*, 1999). It has been suggested that B-lymphocytes may act directly by serving as a "transport vehicle" by delivering prions physically to the nervous system, or indirectly by promoting neuroinvasion through the secretion of factors like antibodies or cytokines, or inducing maturation of FDCs, which may in turn be directly responsible for neuroinvasion (Klein *et al.*, 1998). It appears that the RML scrapie isolate, unlike the ME7 strain, is able to replicate in bone marrow-derived cells, and that different scrapie strains may target different cell types in lymphoid tissue (Brown *et al.*, 1999) as they do in brain tissue (Bruce *et al.*, 1989). Kapasi and colleagues (1998) showed, however, that bone marrow contains FDC precursors and could therefore, be derived from primary lymphoid tissue. Pre-FDCs may leave the bone marrow and become dispersed in tissues throughout the body; the intermediate-type pre-FDC supply could be great enough to provide precursors for months, resulting in development of new FDC networks (Kapasi *et al.*, 1998). In an earlier study (Kapasi *et al.*, 1993), it was found that B- and T-cells can induce host FDC development in SCID mice, suggesting that FDC precursors were present in these mice. Rather than reconstitute the bone marrow with B-lymphocytes, it has been

suggested that SCID mice be reconstituted with various kinds of bone marrow cells including leukocyte precursors.

TNF-R1$^{-/-}$ mice, which apparently lack mature FDCs, were found to be very susceptible to peripherally administered prions (Klein *et al.*, 1997). However, immunomodulation alters susceptibility to TSEs in rodent models (Mabbott *et al.*, 1998) and may be in accordance with the finding that resistance to scrapie of immune-deficient mice may be overcome by relatively large inocula of prions (Brown *et al.*, 1999) and may directly infect peripheral endings bypassing the need for replication in peripheral tissues (Fraser *et al.*, 1996; Lasmézas *et al.*, 1996). It has been suggested that susceptibility of TNF-R1$^{-/-}$ mice to peripherally administered prions may depend on a population of cells that is not identified by the FDC markers, FDC-M1 and FDC-M2 (Klein *et al.*, 1998).

Studies using athymic mice indicated that T-lymphocytes were not involved in TSE pathogenesis (Fraser and Dickinson, 1978; McFarlin *et al.*, 1971). One major point is worth mentioning with respect to these studies. Recently, new GALT has been described in murine (isolated lymphoid follicles or ILFs and "cryptopatches") (Ishikawa *et al.*, 1999; Kanamori *et al.*, 1996) and rat (lymphocyte-filled villi or LFV) (Moghaddami *et al.*, 1998) small intestine. ILFs have been described as being structurally and functionally similar to the follicular units that compose PPs and may serve as a complementary system to PP for the induction of intestinal immune responses. ILFs develop normally in the progeny of transplacentally manipulated PP-deficient mice and decreased numbers of conspicuously atrophied ILFs have been found to be present in IL-7Rα$^{-/-}$ PP^{null} mice (Hamada *et al.*, 2002). Researchers investigating the role of PPs in oral TSE pathogenesis need to take these observations into consideration since previous studies may have examined the role of PPs without considering the compensatory role of ILFs.

Cryptopatches are tiny areas of lymphoid tissue situated randomly around the circumference of the intestinal wall of mice (Hamada *et al.*, 2002), and are not detectable until the fourteenth day post-partum (Kanamori *et al.*, 1996). Murine cryptopatches and rodent LFV occur in athymic (nude) mice and rats, respectively (Moghaddami *et al.*, 1998). Evidence shows that large numbers of T-cells develop in the mucosa of the murine small intestine without passing through the thymus. These T-cells, derived from the intestine, are called intestinal intraepithelial T-lymphocytes (IELs); IELs are concentrated in cryptopatches (Ishikawa *et al.*, 1999). Both cryptopatches and rodent LFV have been identified as sites of

primary extrathymic T-cell differentiation (Moghaddami *et al.*, 1998). The reliability of studies investigating the role of T-lymphocytes in TSE pathogenesis (Fraser and Dickinson, 1978; McFarlin *et al.*, 1971) remains doubtful since extrathymic T-cell development in cryptopatches was not considered. Nonetheless, a more recent study (Klein *et al.*, 1997) using the RML isolate support the finding that T-cell defects do not impair susceptibility to scrapie.

It will be interesting to determine whether the findings described above are also valid for other species, including sheep and non-human primates, using oral inoculation, and whether the development of disease may be accelerated in conditions such as inflammatory bowel disease, whereby, the number of FDC-containing lymphoid aggregates is increased in the intestine (Yeung *et al.*, 2000). Most studies have been directed at splenic FDCs. Perhaps future studies also need to focus on FDCs present in other lymphoid organs, including PPs. Whether the time of appearance of FDCs in lymphoid tissue, including lymphoid follicles of PPs helps to determine susceptibility of an individual to TSE infection is well-worth investigating and forms the basis of this thesis.

1.2 Aims of project

Previous studies using mathematical models suggest that exposure to BSE-contaminated meat and meat products could not explain the young age distribution of vCJD cases in the UK and that an additional effect of age-dependent susceptibility was required to fully account for the age of vCJD cases (Boëlle et al., 2004). Susceptibility to vCJD was predicted to increase during childhood, peak during adolescence and decrease sharply afterwards. Although the oral route appears to be the most likely route of transmission for natural TSE infections, including scrapie in sheep (Heggebø et al., 2000) and BSE in cattle (Terry et al., 2003), little attention has been paid to the gastrointestinal tract and the mechanisms surrounding its involvement in determining age-related susceptibility to risk of oral TSE infection. Whether young individuals may be more susceptible to natural TSE infection because of developmental differences in their gut anatomy has been suggested but remains to be determined.

This study aims to investigate the potential role of GALT in determining age-related patterns in incidence of natural cases of TSEs. The main objectives of this project are outlined as follows:

i) To determine the effect of age at exposure to the TSE agent on susceptibility and incubation period of the disease using the NPU experimental scrapie dataset.

ii) To quantify the number of lymphoid follicles and surface area of ileal PP tissue in the distal ileum of different age groups of Cheviot sheep.

iii) To determine whether a link exists between available measures of PP development and the estimated risk of infection for an individual of the corresponding age using anatomical data and estimates of risk of infection in mathematical models for sheep, cattle and humans.

iv) To investigate the ontogeny of FDCs and associated PrP in ileal PP tissue of postnatal mice and sheep using immunocytochemistry (ICC).

1.3 Overview of thesis

Chapter 2 follows this general introduction (Chapter 1) and employs statistical methods, including logistic regression and survival analyses, to demonstrate the effect of age at exposure on scrapie outcome and incubation period of the disease, respectively. Several other factors related to susceptibility were incorporated into the models simultaneously. Subsequent to this, a quantitative study was performed to determine the amount of PP tissue and the lymphoid follicle density in the distal ileum of different age groups of Cheviot sheep (Chapter 3). The anatomical data obtained from this sheep study were required as part of a comparative epidemiological study (Chapter 4) that uses anatomical PP data and estimates of risk of infection (derived using mathematical models) to provide evidence for a link between PP development and age-related risk of TSE infection in sheep, cattle and humans. Chapter 5 investigates the ontogeny of PrP-associated FDCs in postnatal mice and sheep and examines how the onset of FDC development may be related to age-dependent susceptibility to TSEs in these two species. Finally, in Chapter 6, the results from statistical, mathematical and ICC studies are integrated and interpreted, and implications for future research are discussed.

Chapter 2: Age at exposure contributes to determining the incidence and survival times of sheep experimentally infected with the TSE agent

2.1 Introduction

The link between BSE and vCJD, and the recently confirmed case of BSE in a French goat (Brugere-Picoux *et al.*, 2005) has heightened scientific interest in oral transmission of the TSEs and has refocused efforts to control scrapie. The majority of TSE cases occurs in sheep aged 2-4 years (Baylis and Goldmann, 2004). This is in comparison to other natural cases of TSEs, including BSE and vCJD (Arnold and Wilesmith, 2004; Will *et al.*, 2000) where most cases occur in adolescents and young individuals. The reasons for the current age distribution of TSE cases are today, speculative (Ghani *et al.*, 1998) but may be partly explained by a higher susceptibility of younger animals to TSE infection (Diaz *et al.*, 2005; Matthews *et al.*, 2001).

In the ovine PrP gene, three codons at positions 136 (A, V), 154 (R, H) and 171 (R, Q, H) largely influence the incidence and age of onset of natural and experimental scrapie (Houston *et al.*, 2002; Hunter, 1997b; Hunter, 2003). In many breeds, such as Cheviot, the VRQ allele and the ARR allele have consistently been associated with the highest susceptibility or natural resistance to clinical disease in the field, respectively (Goldmann *et al.*, 1994; Hunter *et al.*, 1996; Hunter *et al.*, 1997; Hunter *et al.*, 2000).

Generally, sheep of genotypes of greatest scrapie risk (VRQ/VRQ and ARQ/ARQ) die, on average, at the youngest age (Baylis and Goldmann, 2004). In natural scrapie, this manifests as a difference in the mean ages at death from scrapie for sheep of different PrP genotypes (Baylis *et al.*, 2002; Bossers *et al.*, 1996). Although host genotypic differences explain much of this variation, they do not account for all of the observed variation in incubation period nor do they explain why animals with susceptible genotypes do not always develop disease.

Several studies suggest that a number of other genes may influence the scrapie incubation period in inbred lines of mice (Manolakou *et al.*, 2001) or sheep (Moreno *et al.*, 2002), and that non-genetic factors may also be involved in the control of the disease (Elsen *et al.*, 1999; Manolakou *et al.*, 2001). In sheep, it has been shown that interactions exist between host genotype and scrapie strain (Goldmann *et al.*, 1994; O'Rourke *et al.*, 1997). Using several different strains of natural scrapie in mice, the incubation period of the disease was found to be associated with PrP genotype (Moore *et al.*, 1998) and with the prion strain inoculated

(Bruce *et al.*, 1991). In general, the incubation period is lengthened when a smaller dose of agent is administered (Bruce *et al.*, 1991; McLean and Bostock, 2000) or when a peripheral route of inoculation is employed instead of an i/c route (Bruce *et al.*, 1991). One study showed that the risk of oral infection of scrapie in mice depended not only on the total dose, but whether the dose was given as one or a series of challenges (Gravenor *et al.*, 2003).

Because the oral route is the most likely route of natural transmission of scrapie and vCJD, information about scrapie may be helpful in understanding the current epidemiological trend of vCJD. However, it is important to remember that extrapolations to vCJD must take into account the additional complexity of disease development including, for example, the infectious agent, the host's environment and the species involved as source and recipient of the infectious agent (species barrier). So far, it is not known whether the age at which sheep become infected determines to any extent the incubation period of scrapie, or the likelihood of an animal developing clinical signs, and whether this factor may help to explain the current age distribution of scrapie and other natural cases of TSEs.

The aim of this study is therefore, to determine the influence of age at exposure on incidence and incubation period of scrapie/BSE (henceforth, referred to as TSEs), using data from an experimental NPU Cheviot flock, and taking into account the effect of several other factors including genetic susceptibility, strain, route and dose of agent.

2.2 Methods

This study comprises data from experiments carried out on NPU Cheviots from 1960 to 2002. These experiments were designed to study, by classical genetic breeding experiments, the well-established incubation period differences in family lines of the sheep, and to create 'susceptible' and 'resistant' lines of NPU Cheviots (henceforth, referred to as positive and negative selection lines, respectively) based on differing incubation periods following inoculation with a source of scrapie called SSBP/1 (Scrapie Sheep Brain Pool number 1) (Dickinson, 1976). Cheviots that developed disease from s/c inoculation in 150-400 days were bred as the positive line and those animals, which showed no clinical signs of disease within their normal lifespan, were grouped in the negative line. Polymorphisms of the PrP gene have been found to be associated with differences in artificially induced susceptibility. It has since been discovered that the two lines of sheep differ in PrP genotype frequencies, the positive line animals encoding at least one copy of the VRQ allele and the negative line

animals being AXX/AXX (Hunter *et al.*, 1996). The origin and history of the experimental NPU flock is described in greater detail elsewhere (Hunter, 1997a; Hunter *et al.*, 1996).

This study was based on experiments that commenced in 1960 and comprises several different experiments. Although experiments were performed with many different combinations of sheep breeds, TSE strains (including BSE), doses and routes of inoculation and, therefore, cannot be directly compared on the basis of incubation period or development of TSE, the method used here of combining data from many different experiments allows the assembly of such information from existing data archives without committing unreasonable resources to new experimentation. Similar type datasets have been used to investigate the effect of several variables on infection probability and incubation period. By adopting the concept of relative dose (that is, relative to ID_{50}), McLean and Bostock (2000) were able to standardize dosing across 117 different titration experiments in the murine scrapie model thus creating a dataset of sufficient size to address questions on effect of age, sex and dose on infection probability and incubation period of scrapie; these experiments comprised over 4,000 animals and encompassed over 30 years of experimental work. (See McLean and Bostock (2000) for full explanation on method used for calculating ID_{50}s).

2.2.1 Data Management

Experiments were performed between 1960 and 2002 on 2367 NPU sheep. All sheep which were challenged came from the breeding flock. Animals for injection were moved to an experimental shed where work with experimental TSE had been conducted for many years (Dickinson and Stamp, 1969). Sheep breeds included the Cheviot, Herdwick, Swaledale and Cheviot-Herdwick cross. Goats were also used in the experimental studies.

This present study comprises a subset of this experimental dataset and includes only purebred Cheviots with birth dates ranging from January, 1960 to April, 2002. For each animal, data included an identification number, sex, date of birth, date of exposure to infectious agent, selection line, dose and route of inoculation used, strain of agent and date of death or onset of clinical signs. Confirmation of suspect TSE cases through histopathological detection of vacuolation of brain tissue was available for 1372 sheep. Animals were not always kept until their natural death but were culled if they developed any form of debilitating, intercurrent disease. An animal was also culled if it was suspected of having TSE based on clinical signs.

2.2.2 Description of study variables

The different factors included in the models are as follows.

2.2.2.1 Age at exposure (age_exp).

Sheep, with ages ranging from 1 to 3070 days, were classified into two age categories: 0-18 months and >18 months. This grouping was based on epidemiological observations, which suggest that young ruminants are more susceptible to TSE infections than older ones (Detweiler and Baylis, 2003).

2.2.2.2 Dose/route of exposure (d/r).

This factor represents a combined effect of dose and route of exposure. Five levels of the d/r of exposure effect were defined: i/c injection given at a low dose (0.3, 0.35 or 0.5 ml per animal); i/c injection given at a high dose (0.9 or 1.5 ml per animal); s/c injection given at a low dose (0.5, 1.0, 1.5 or 2.0 ml per animal); s/c injection given at a high dose (5 or 15 ml per animal); and oral infection using 50 ml per animal. I/c and s/c challenges were carried out using a 10^{-1} dilution of brain homogenate. For oral infection, a dose of 50 ml of a 10% BSE cow brain homogenate (equivalent to 5 g of infected brain tissue) was used.

2.2.2.3 Strain of agent (agent).

This factor represents the strain of agent used for inoculation. Four levels of agent were defined: BSE, CH1641, Half-bred and SSBP/1 (the latter three being sheep-passaged TSE strains). SSBP/1 has been transmitted by injections of brain material in Cheviot sheep for 25 passage-generations. In some of the passages Scottish Blackface and Half-bred sheep brains were included as minor constituents of the pool of this agent (Dickinson and Stamp, 1969).

2.2.2.4 Selection line (seln_line)

Genotype information at the PrP gene locus was available for only 154 sheep. Information on selection lines was more complete, and was therefore expected to provide additional information. 197 animals had no selection line (n = 156) or were classified as heterozygotes (n= 41). These animals were included in the analyses to obtain a better estimate of other risk factors in the model. Hence, three levels of this variable (seln_line) were defined: The positive selection line, the negative selection line and other.

2.2.2.5 Definition of cases

For purposes of analysis, sheep were identified as 'TSE animals' when they showed clinical signs (including pruritus and incoordination of gait) and/or positive histology. Hence, suspect TSE cases were not always confirmed by histopathological detection of vacuolation of brain tissue. Natural scrapie was first recognised in the NPU Cheviot flock in 1968 (Dickinson, 1974) and only affects sheep of the VRQ/VRQ and VRQ/ARQ genotype. Therefore, if scrapie occurs in these genotypes with an incubation period of less than 150 days (inoculation of SSBP/1 in VRQ/VRQ Cheviot sheep results in the shortest incubation periods, on average of about 150 days) then cases are always considered to be natural scrapie (Hunter, 2003; Nora Hunter, personal communication). For purposes of analyses, animals that showed clinical signs of TSE or died and had positive histology at less than 150 days following challenge were excluded from the analyses since they may have been preclinically infected through natural exposure rather than through experimental inoculation.

2.2.3 Statistical Analyses

Descriptive statistics were performed on all variables entered in the model. All statistical analyses were performed using S-PLUS 2000 for Windows (Mathsoft Inc., USA).

2.2.3.1 Logistic Regression

Conditional logistic regression was used to evaluate the effect of age_exp in 1505 sheep after adjusting for seln_line, agent and d/r on TSE status ('TSE' or 'no TSE'). First, a univariate analysis of each variable was performed. Variables significant in the univariate analysis at the 25 percent level (based on the chi-square test) were eligible for inclusion in the model. The model building process began with a multivariate model containing all variables significant in the univariate analysis. Variables in the model that were not themselves significant ($P < 0.05$) and did not improve the overall fit of the model (as determined by likelihood ratios) were rejected. As a final check, all rejected variables were entered individually into the final model to determine whether they became significant or produced an "important" change (of about 20 percent) in the coefficients of other variables already included in the model. Seln_line was always added first to the model because of the known effect of genotype on TSE outcome. Age_exp was added to the model last in order to obtain an estimate of the additional effect of this variable, adjusting for other significant covariates. Two-way interaction terms were selected from the main effects in the model. The significance of each separate interaction was assessed by adding it to the main effects model and using the likelihood ratio test statistic (for the current model versus the main effects

model), the degrees of freedom for this test and its P-value. All interactions significant at the 5 percent level were added jointly to the main effects model. Since the 'd/r x age_exp' interaction was of major interest it was added last to the model. The model fit was assessed using deviance residuals.

2.2.3.2 Survival Analysis

Survival analysis is suitable for experimental studies when the time having been at risk is known for each individual. The results of this type of analysis can also be seen as a refinement of results of logistic regression, where the occurrence ('yes' or 'no') of the event is analysed. In this study, survival analysis was used to model the hazard for an animal to show signs of TSE (or death due to TSE) in an interval after time *t*, conditional on the subject not being affected until then. The measure of the effect obtained is called a hazard ratio (HR) and is expressed in terms of an exponential of a regression coefficient in the model. Failure time was defined as the time between which an animal was infected and the date that it developed clinical signs of TSE, or was culled (as a result of intercurrent disease and without showing clinical signs of TSE) and later confirmed as TSE positive from brain histology. From henceforth, failure time is referred to as the incubation period or 'time till death'. In this study, a cut-off point of six years was used since most/all animals are expected to have developed experimental TSE by then. This resulted in the loss of 263 observations. In the 1960s, a large number of sheep were culled on the same day after being challenged with infectious agent. Because this would have biased estimates of survival times of these sheep they were excluded from this part of the study. Consequently, a further 661 observations were lost. As a result the total number of animals used in the survival analysis was 581. Of these, 240 did not develop TSE and were recorded as censored observations. Animals which were still alive at the time of the analysis and showed negative pathology for TSE or animals that died from causes other than TSE (intercurrent deaths) were also included as censored observations. Intercurrent deaths were recorded as censored observations and occurred if: an animal died with clinical signs of TSE and had a negative pathology result; died with no clinical signs of TSE and had a negative pathology result; or died with no clinical signs of TSE and had no recorded pathology. Mistaken culls occurred when an animal showing clinical signs was killed and had a negative pathology result; mistaken culls were also recorded as censored observations.

Model building began with a bivariate (non-parametric) analysis of the association between survival time and the variables, age_exp, seln_line, agent and d/r. Median survival times

(±95% confidence intervals) were obtained and differences between survivorship curves were tested using the Kaplan-Meier estimator and the log-rank test. Significance was set at $P < 0.05$. Cox proportional hazard models were used to determine the effect of age_exp on survivorship period after adjusting for significant covariates. A multivariate model containing all variables significant in the bivariate analysis was run. The variable age_exp was always added to the model last. P-values from the Wald tests of the individual coefficients were used to identify covariates that might be deleted from the model. Any variable excluded from the initial multivariate model was added back to the model to confirm that it was neither statistically significant nor an important confounder. How well the fit of a model improved when variables were removed or added to it was assessed by observing changes in the log-likelihood values. Two-way interaction terms were selected from the main effects in the model using P-values from the likelihood ratio test as a guide to selecting interactions. The proportional hazard (PH) assumption, which assumes that the HRs (that is, the conditional relative risks across substrata) are proportional to one another and this proportionality is maintained over time was assessed numerically and visually using scaled Schoenfeld residuals (Therneau and Grambsch, 2000). In the final model, adjusted HRs and 95% confidence intervals (CIs) were reported.

2.3 Results

2.3.1 Logistic Regression

This dataset comprised a total of 1505 sheep (Table 2.1). The factors seln_line, agent, d/r and age_exp were found to be associated with disease/TSE outcome. Although agent was significant in the univariate analysis (Table 2.2), it became non-significant (with the exception of agent3) when combined with the other variables in the multivariate logistic regression model (Table 2.3). However, it was left in the model because of its confounding effect on d/r and age_exp. All levels of the variables, age_exp and seln_line, were found to be significant both in the univariate and multivariate models. Seln_line was strongly associated with TSE outcome ($P < 0.001$) and made the largest contribution to explain differences in risk among animals. Animals belonging to the positive selection line were about 8 times more likely of being TSE-positive compared to animals in the negative selection line. There were differences associated with some levels of the variables, agent and d/r (Table 2.3). Sheep that were challenged with BSE were at an increased risk of being TSE-positive (OR: 5.518; CI: 1.307-23.303) compared to animals infected with the Half-bred strain. S/c injection at low dose (d/r3), and oral exposure decreased the risk of developing TSE (OR: 0.406; CI: 0.235-0.703 and OR: 0.172; CI: 0.060-0.488, respectively)

relative to the i/c, low dose (d/r1) route. Older animals (> 18 months) had an almost 30% lower chance of being TSE positive than younger animals (0-18 months) ($P < 0.01$). All possible two-way interactions (including the'd/r x age_exp' interaction) were found to be significant on their own (Table 2.4). When the 'd/r x age_exp' interaction was added last to the main effects model plus all other interactions found to be significant at the 5 percent level, it became non-significant ($P = 0.098$).

Table 2.1 Description of the risk factors for TSE in the experimental NPU Cheviot flock (n = 1505) used in the logistic regression.

Variable	Category	Status		Total number of observations
		TSE	No TSE	
age_exp 1	< 18 months	251	203	454
2	18 + months	454	597	1051
seln_line 1	negative	125	485	610
2	positive	463	235	698
3	other	117	80	197
agent 1	Half-bred	5	13	18
2	CH1641	43	93	136
3	BSE	18	22	40
4	SSBP/1	639	672	1311
d/r 1	ic, low dose	66	91	157
2	ic, high dose	6	10	16
3	sc, low dose	601	653	1254
4	sc, high dose	20	15	35
5	oral	12	31	43

Table 2.2 Univariate logistic regression models (n = 1505) for NPU experimental TSE dataset. Significant results are indicated in red.

Variable	Coefficient (β)	Odds ratio (OR)	95% CI	P-value
Constant	-0.126			
age_exp2	-0.486	0.615	0.493-0.768	< 0.001
d/r2	-0.190	0.827	0.286-2.388	< 0.01
3	0.238	1.269	0.907-1.774	
4	0.609	1.839	0.876-3.857	
5	-0.627	0.534	0.256-1.116	
agent2	0.184	1.202	0.403-3.581	< 0.001
3	0.755	2.128	0.639-7.088	
4	0.905	2.472	0.876-6.972	
seln_line2	2.034	7.645	5.948-9.825	< 0.001
3	1.736	5.675	4.019-8.012	

Table 2.3 Estimated coefficients, estimated standard errors and P-values for the multivariate model containing variables (where P < 0.25) identified in the univariate analysis. Significant results are indicated in red.

Variable	β	SE(β)	OR	95% CI	P-value	Change in deviance	df
Constant	-1.253	0.677					
seln_line2	2.101	0.138	8.174	6.262 – 10.671	< 0.001	303.773	2
3	1.792	0.184	6.001	4.201 – 8.574			
agent2	0.419	0.702	1.520	0.410 – 5.642	0.245	4.154	3
3	1.708	0.777	5.518	1.307 – 23.303			
4	0.947	0.711	2.578	0.688 – 9.660			
d/r2	-0.051	0.610	0.950	0.287 – 3.147	0.001	17.890	4
3	-0.910	0.289	0.406	0.235 – 0.703			
4	-0.312	0.478	0.732	0.293 – 1.832			
5	-0.762	0.575	0.172	0.060 – 0.488			
age_exp2	-0.344	0.129	0.709	0.551 – 0.913	0.007	7.157	1

Log-likelihood: -2285.461

Table 2.4 P-values for interactions, added individually, to the main effects model. Significant results are indicated in red.

Interaction		P-value	Change in deviance	Log-likelihood
seln_line	x agent	< 0.001	22.990 on 5 df	-2279.785
	x d/r	< 0.001	46.050 on 7 df	-2299.576
	x age_exp	< 0.001	22.913 on 2 df	-2283.758
agent	x d/r	< 0.01	19.653 on 6 df	-2312.211
	x age_exp	< 0.05	9.593 on 3 df	-2286.851
d/r x age_exp		< 0.05	12.470 on 4 df	-2288.927

2.3.2 Survival Analysis

A total of 581 sheep were used in this analysis (Table 2.5). 240 animals were found to be TSE negative and 341 were TSE positive. Data showed a long-tailed survival distribution (Figure 2.1), with the majority of TSE deaths occurring around 300 days. There was a significant difference in the mean ages at death for animals that died from TSE (341/581) and animals that died from other (non-scrapie) causes (240/581) (two sample t-test, t = 18.139 on df = 579; P = <0.0001. Data (time in days) was log transformed for non-normality. (The detransformed means were as follows: For animals with no TSE (TSE = 0), mean = 824.1; 95% CI: 755.1 – 899.5). For animals with TSE (TSE = 1), mean = 345.1; 95% CI: 328.9 – 363.1). There was a significant difference (Chi-square (X^2) = 10.832 on 1 df; P = 0.001) in the number of intercurrent deaths between young (55/224 or 24.6%) and old animals (136/357 or 38.1%).

Table 2.5 Estimated median times to development of TSE/death due to TSE, with 95% CIs and log-rank test P-values for categorical variables in the NPU experimental TSE study (n = 581). Significant results are indicated in red.

Variable		Category	No. of observations	Median time (95% CI)	Log-rank test P-Value
age_exp	1	< 18 months	224	321 (298, 343)	< 0.001
	2	18 + months	357	691 (500, 1176)	
seln_line	1	negative	168	2085 (2027, NA)	< 0.001
	2	positive	363	362 (348, 377)	
	3	other	50	312 (275, 338)	
agent	1	Half-bred	18	NA, (1140, NA)	< 0.001
	2	Ch1641	111	2085 (2085, NA)	
	3	BSE	32	912 (663, 1925)	
	4	SSBP/1	420	361 (343, 376)	
d/r	1	ic, low dose	122	1886 (1176, NA)	< 0.001
	2	ic, high dose	14	NA (464, NA)	
	3	sc, low dose	389	362 (344, 376)	
	4	sc, high dose	19	305 (234,942)	
	5	oral	37	1701 (912, NA)	

NA: No output provided

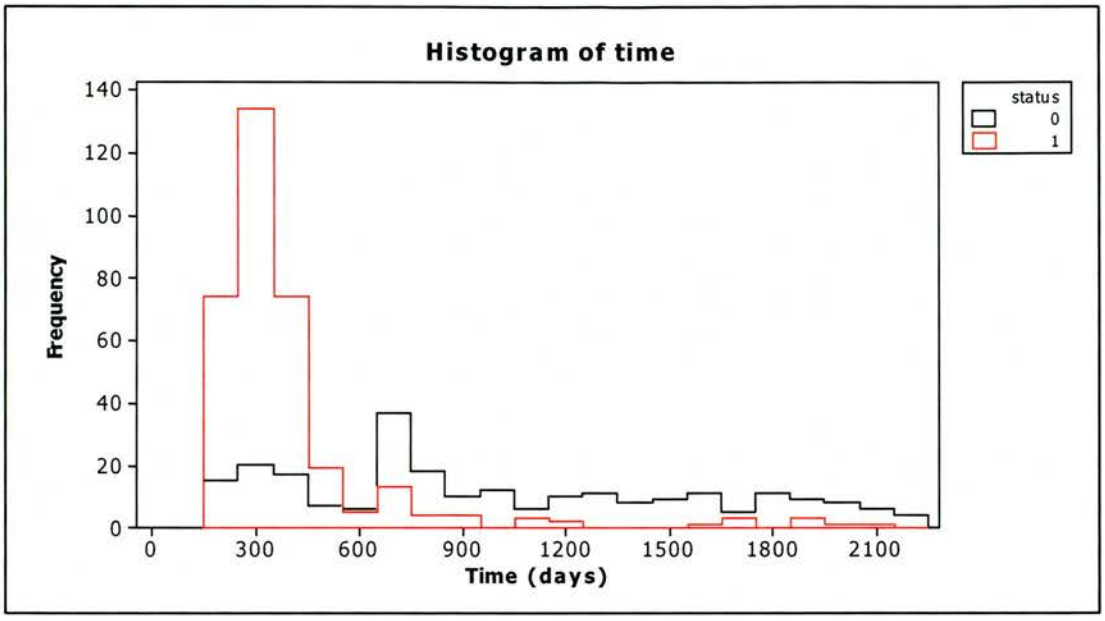

Figure 2.1 Histogram showing distribution of data used in the survival analysis. Status represents 'TSE outcome': 0 = censored observation; 1 = TSE.

All variables were found to be significant ($P < 0.05$) in the bivariate analysis (Table 2.5). The PH assumption was tested numerically (Table 2.6) and smoothed scaled Schoenfeld residual plots for the predictors (Figure 2.2(A-J)) provided a visual interpretation of the non-proportionality. The PH assumption did not hold for age_exp, the impact of age_exp clearly changing with time (Figure 2.2J). Because of this violation, a piecewise Cox regression was used. This involved partition of the time axis at a time point suggested by the residual plots and the median of the event times (375 days), and running separate Cox regressions on the two subsets of the data (Therneau and Grambsch, 2000). Estimates for the piecewise Cox regression are presented in columns 3 and 4 of Table 2.7. Although age_exp could have been incorporated into the model as a stratified variable, this method does not provide an estimate of the impact of the stratified variable (in this case, age_exp, the main variable of interest) on the hazard rate. Estimated HRs in the multivariate analysis were found to be significant for selection line, BSE and age_exp ($P < 0.05$) (Table 2.8). HRs of above one were obtained for seln_line2 and seln_line3 for each piecewise Cox regression, indicating that incubation period/'time till death' was considerably shorter compared to the negative selection line (seln_line1) over the entire study.

Table 2.6 Testing the PH assumption using statistical tests for significant slope in the plots shown in Figure 2.2.

Variable	rho	Chi-square	P-value
seln_line2	-0.049	0.551	0.458
seln_line3	0.024	0.173	0.677
agent2	-0.103	4.115	0.043
agent3	-0.027	0.232	0.630
agent4	-0.104	3.945	0.047
d/r2	-0.032	0.354	0.552
3	-0.156	4.972	0.026
4	-0.068	1.315	0.251
5	-0.015	0.090	0.764
Age_exp	0.318	33.914	<0.001

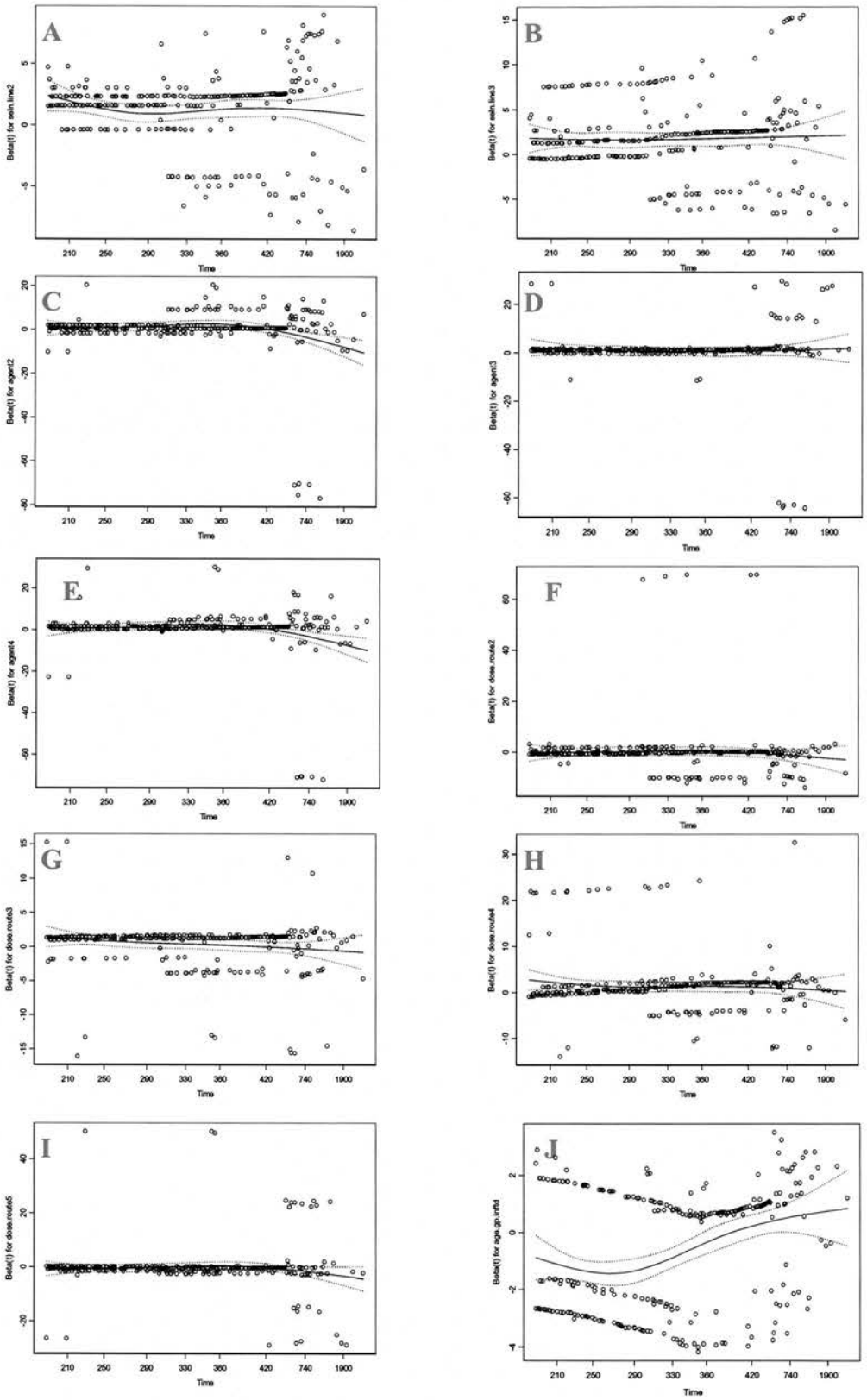

Figure 2.2 Graphs of the scaled Schoenfeld residuals to assess the PH assumption for variables in the main effects model (see Table 2.6) for **A**. seln_line2; **B**. seln_line3; **C**.agent2; **D**. agent3; **E**. agent4; **F**. d/r2; **G**. d/r3; **H**. d/r4; **I**. d/r5 and **J**. age_exp.

Table 2.7 Cox PH results for NPU experimental TSE data.

Variables	Coefficient (β) SE(β)	Piecewise Cox Regressions (Coefficients)	
		Time < 376	Time ≥ 376
seln_line2	1.297**	1.104**	1.699**
	(0.191)	(0.425)	(0.315)
seln_line3	1.758**	1.017**	1.454**
	(0.233)	(0.473)	(0.440)
agent2	0.541*	1.065*	-0.427*
	(0.499)	(1.019)	(0.603)
agent3	0.917**	2.118**	1.229**
	(0.519)	(0.917)	(0.531)
agent4	0.658*	NA	-0.614*
	(0.508)	NA	(0.643)
d/r2	-0.107*	0.309*	-0.469*
	(0.483)	(0.625)	(0.799)
3	0.434**	0.676*	0.168*
	(0.224)	(0.954)	(0.423)
4	1.399**	1.061*	0.255*
	(0.326)	(0.982)	(1.044)
5	-0.444*	-0.529*	-0.685*
	(0.393)	(1.190)	(0.476)
age_exp	-0.640**	-0.825**	0.824**
	(0.115)	(0.145)	(0.282)
Likelihood ratio	212 on 12 df**	60.7 on 9 df**	65 on 10 df**
N	581	289	292

*: P < 0.10; **: P < 0.05; N = number of observations; df = degrees of freedom; NA=no output provided.

However, the impact of selection line appeared to be greater for longer incubation periods (time ≥ 376 days). The agent "BSE" had a HR of about eight times that for the reference level ("Half-bred") for time < 376 days, with the impact of this agent decreasing for longer incubation periods (HR = 3.420) (for time ≥ 376 days). A HR of 0.438 was obtained for older animals for time < 376; this indicated that older sheep developed TSE at a rate that was about 56% lower than for younger sheep for this time period (Table 2.8). The negative impact of the variable, age_exp, appears to be driven by shorter rather than longer incubation periods, and there is a complete reversal of sign in the coefficient for age_exp for time ≥ 376 days, with older animals having a HR about twice that for younger animals (Table 2.8). Coefficients (for age_exp) attained statistical significance in the piecewise Cox models (P < 0.05) (see Table 2.8).

Table 2.8 HRs and 95% CIs and P-values for the Cox PH model containing variables significant at the 25% level in the bivariate analysis in the NPU experimental TSE study (n = 581). Significant results are indicated in red.

Variable	HR (95% CI for HR)		Wald Statistic P-Value	
	Time < 376	Time ≥ 376	Time < 376	Time ≥ 376
seln_line2	3.016 (1.311-6.940)	5.467 (2.947-10.140)	< 0.01	<0.001
3	2.764 (1.094-6.986)	4.280 (1.805-10.150)	< 0.05	<0.001
agent2	2.900 (0.394-21.363)	0.652 (0.200-2.130)	0.300	0.480
agent3	8.315 (1.377-50.198)	3.420 (1.208-9.680)	<0.05	<0.05
agent4	NA NA	0.541 (0.154-1.910)	NA	0.34
d/r2	1.362 (0.400-4.635)	0.625 (0.136-2.880)	0.620	0.470
3	1.966 (0.303-12.746)	1.183 (0.516-2.710)	0.480	0.690
4	2.890 (0.422-19.810)	1.291 (0.167-10.000)	0.280	0.810
5	0.589 (0.057-6.072)	0.504 (0.198-1.280)	0.660	0.150
age_exp2	0.438 (0.330-0.582)	2.280 (1.312-3.960)	< 0.001	<0.01

NA: No output provided

Table 2.9 Testing the PH assumption, numerically, for the final model.

Variable	rho		Chi-square		P-value	
	Time < 376	**Time ≥ 376**	**Time < 376**	**Time ≥ 376**	**Time < 376**	**Time ≥ 376**
seln_line2	-0.107	-0.097	1.426	0.809	0.232	0.368
seln_line3	-0.028	0.054	0.119	0.310	0.731	0.578
agent2	0.057	-0.153	0.644	3.020	0.422	0.082
agent3	-0.090	0.0	4.074	0.0	0.050	0.999
agent4	NA	-0.128	NA	1.81	NA	0.179
d/r2	0.027	-0.080	0.180	0.682	0.671	0.409
3	0.023	-0.107	0.121	0.871	0.728	0.351
4	0.036	0.009	0.285	0.007	0.594	0.933
5	0.099	-0.072	3.151	0.545	0.076	0.460
age_exp	0.069	0.034	1.177	0.099	0.278	0.752

NA: No output provided

In the final model, there was no evidence of non-proportionality as none of the P-values were found to be significant at the conventional 0.05 level (Table 2.9). Although partitioning of the time axis was effective in removing the problem of non-proportionality, the disadvantage is that the power of the study is less because of fewer time events. Interaction terms were not included in the final model because it was uncertain as to whether the model, comprising 581 cases and four factors (most comprising several levels) could handle all interactions successfully. However, because the 'd/r x age_exp' interaction was of interest in this study, to view a non-parametric estimate of the age_exp effect after adjusting for possible differences in d/r distributions, plots of the stratified fit were produced as shown in Figure 2.3 and Kaplan-Meier estimates (at $P < 0.05$) used to determine whether the two age groups for each route had equal survivor functions. For illustrative purposes, these effects have been summarized graphically across the entire time frame (time < 376 and time ≥ 376). There appeared to be a significant difference between the two age groups for 's/c, low dose' (dr3) route ($P < 0.001$) and 's/c, high dose' (d/r4) route ($P < 0.01$), with younger animals having shorter incubation periods. All young animals developed TSE prior to 350 days when challenged with a high dose of TSE agent via the s/c route compared to 50% in older animals. (However, this difference was not significant: Fisher's exact test: $P = 0.057$). When challenged with a low dose of agent subcutaneously, the majority (93%) of young and about half (47%) of old animals developed TSE prior to 350 days (with a significant difference between young and old animals: Chi-square (X^2) = 63.934 on 1 df; $P = <0.0001$). The effect of age_exp was found to be non-significant for both the i/c and oral routes ($P > 0.05$).

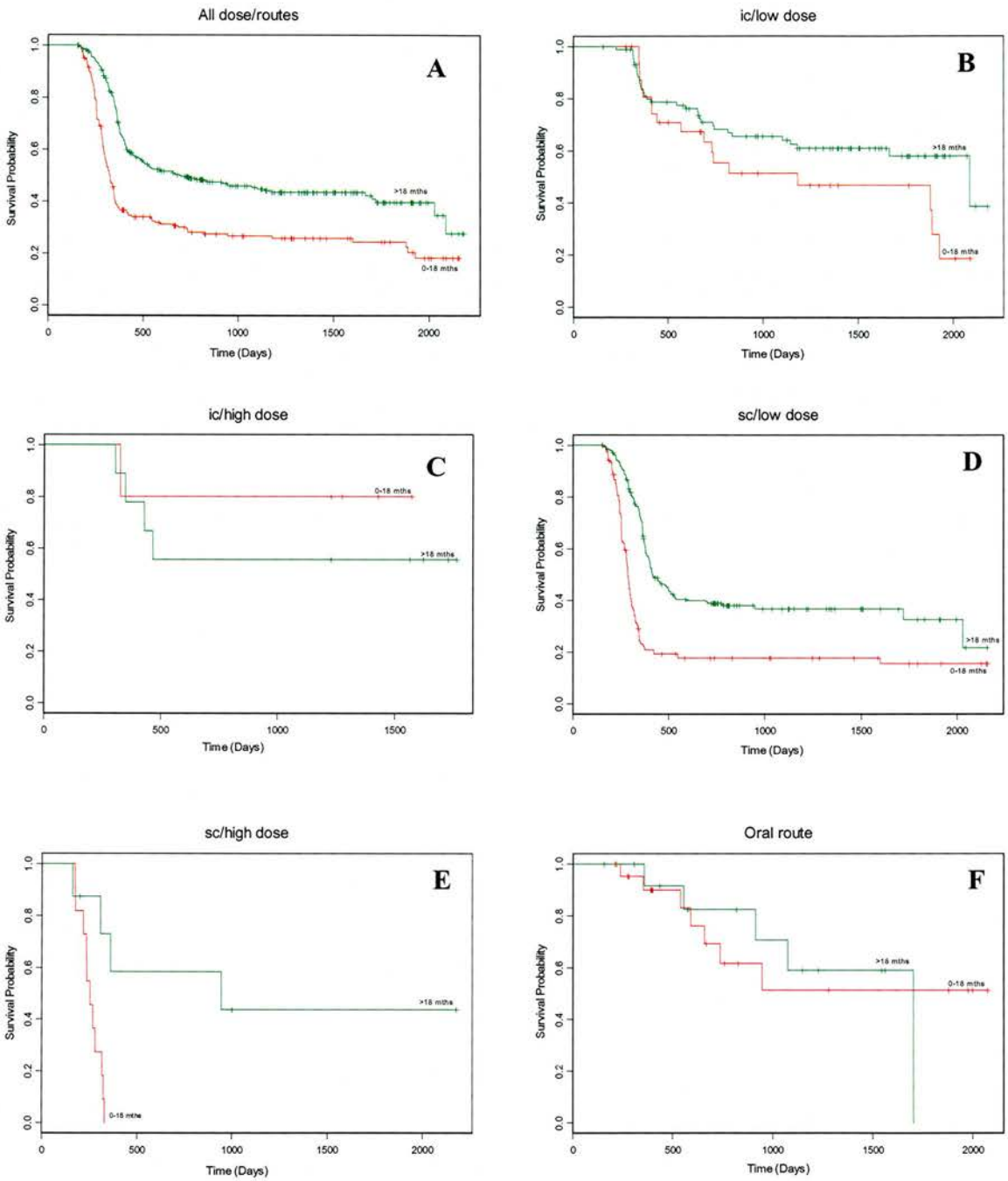

Figure 2.3 Kaplan-Meier plots for the different routes of inoculation, and P-values indicating whether the two different age groups have equal survivor functions. **A**. all doses/routes, $P < 0.0001$; **B**. ic/low dose, $P=0.115$; **C**. ic/high dose, $P=0.436$; **D**. sc/low dose, $P < 0.0001$; **E**. sc/high dose, $P < 0.01$; **F**. oral, $P=0.924$

2.4 Discussion

Variation in susceptibility to TSE can be evaluated by the mortality rate and the incubation period of the disease (Diaz *et al.*, 2005). This present experimental study investigated the effect of age at infection on TSE outcome and incubation period/'time till death', taking into account the effects of several other variables. Experimental challenges revealed a complex relationship between selection line, age at infection, the strain of agent and the dose/route of exposure. This is in agreement with results obtained from previous studies.

2.4.1 Effect of selection line

Sheep from the positive selection line appeared to have a higher risk of becoming TSE animals and had a higher rate of developing the disease than sheep from the negative selection line. Because selection lines were used in this analysis, the results obtained cannot be directly compared to results of other studies, which have used genotype information. However, the two lines of sheep differ in PrP genotype frequencies with positive line animals encoding at least one copy of the VRQ allele and negative line animals being AXX/AXX. Thus, VRQ/XXX sheep appeared to have a higher risk for becoming TSE positive than sheep with the AXX/AXX genotype. The association of TSE status and incubation period with selection line was expected since susceptibility to experimental TSE in NPU Cheviots has been shown to be controlled mainly by host genetics (Goldmann *et al.*, 1994); generally, the shortest incubation periods are associated with the most susceptible genotypes (Baylis and Goldmann, 2004). This assumption has been a requirement for successful modelling of a within-flock scrapie epidemic (Matthews *et al.*, 2001). Other studies in sheep have shown that the average incubation period differs among different genotypes for Cheviots and Dorsets that were infected subcutaneously with SSBP/1 (Houston *et al.*, 2002). In mice, a relationship also exists between the incubation period and PrP genotype (Moore *et al.*, 1998).

Although selection line made the largest contribution to explain the differences in risk among animals, the survival of a number of sheep from the positive selection line and the wide variation of 'incubation times/times till death' of TSE-affected sheep in this present study suggest that factors other than host genetics may also govern the risk of infection. The frequencies of PrP genotypes observed in scrapie-free populations of sheep (Bossers *et al.*, 1996; Hunter and Cairns, 1998) provide evidence that scrapie is not simply a genetic disease, and several studies provide initial estimates for the relative risk of developing the disease taking into account non-genetic factors (Diaz *et al.*, 2005; Elsen *et al.*, 1999; Healy *et al.*,

2004). It has been suggested that additional polymorphisms within the PrP open reading frame or in the flanking regions may modulate incubation times or influence susceptibility (Hunter *et al.*, 1996). One study in a Romanov flock has shown that polygenic variance was responsible for 21% of the total genetic variability that was related to susceptibility to scrapie and that this variation may be important in understanding the existing genetic susceptibility in many sheep populations (Diaz *et al.*, 2005). Although there is evidence that other genes may influence the incubation period of TSEs in inbred lines of mice (Manolakou *et al.*, 2001; Moreno *et al.*, 2003), little is known of their nature and function.

2.4.2 Effect of agent

Susceptibility to ovine TSE is controlled by a combination of host genetics (Belt *et al.*, 1995; Clouscard *et al.*, 1995; Hunter *et al.*, 1992; Laplanche *et al.*, 1993) and the TSE strain used to infect the host (Dickinson and Meikle, 1971; Goldmann *et al.*, 1994). In this present study, different strains of TSE were found to have different incubation periods/'times till death'. Animals infected with BSE had an increased risk of being TSE-positive and developed the disease at a rate that was over three times that for animals infected with the Half-bred strain. This finding demonstrates the degree of pathogenicity of BSE relative to other strains used in this study. However, the amount of BSE material that constitutes an oral infectious dose for human beings and the incubation periods resulting from such exposure are unknown, although this uncertainty has been partly addressed (Herzog *et al.*, 2004; Lasmézas *et al.*, 2005). Several strains of natural TSE have been identified on the basis of their incubation periods under standard conditions of experimental infection (Bruce *et al.*, 1994; Bruce *et al.*, 2002), and some strains appear to attack genotypes differently. In NPU Cheviot sheep, although the ARQ/ARQ genotype is considered to be resistant to scrapie following challenge with SSBP/1 (Goldmann *et al.*, 1994), this genotype has been shown to be susceptible to the CH1641 isolate of scrapie in sheep and goats (Foster *et al.*, 2001). An important limitation of this present analysis was the absence of genotype information and the inability to examine the effect of specific strains on different genotypes. There was, however, a significant interaction between selection line and strain of agent, implying that the effect of agent on TSE outcome varied for the different selection lines.

2.4.3 Effect of dose/route

The oral route of infection was associated with decreased odds of TSE-positive status and animals infected via this route developed TSE at a lower rate compared to the i/c and s/c routes of infection. Evidence from experimental infections in mice indicates that the

incubation period is a function of the route of infection and the infective dose (Bruce *et al.*, 1991). Previous studies have shown that BSE is transmitted more efficiently to sheep (and goats) by i/c inoculation than by oral dosing with the oral route nearly always resulting in longer incubation periods than the i/c route (Foster *et al.*, 2001). Oral or intragastric infection of TSE was found to be about 40,000 times less efficient than the i/c route in mice (the highest effective titre by the intragastric route being 4.3 $\log_{10}$ LD_{50} units/g and the average titre of 139A scrapie brain being 8.9 $\log_{10}$ LD_{50} units/g for the i/c route) (Kimberlin and Walker, 1989a) while a difference as high as 10^9 has been reported in hamsters, using 263K scrapie brain (Prusiner *et al.*, 1985). Although the oral route is relatively inefficient it is believed to be the most likely route of entry for natural TSE infections including scrapie, BSE, CWD, TME, Kuru and vCJD (Andréoletti *et al.*, 2000; Gajdusek, 1977; Hadlow *et al.*, 1982; Marsh and Bessen, 1993; Shmakov and Ghosh, 2001; Williams and Young, 1980). Also, it is not known whether the TSE agent may behave differently in natural disease and may be spread more efficiently via peripheral routes. Although oral inoculation studies of TSEs, including BSE, have been performed in recent years in sheep (Foster *et al.*, 2001; Jeffrey *et al.*, 2001b; O'Rourke *et al.*, 1997), comparisons between them are difficult because of differences in method of infection.

Sheep challenged with high doses of the infectious agent via the s/c route succumbed to TSE after shorter periods than when exposed to lower doses of the agent. For any given route the dose-response curve has been shown to be consistent, that is, the higher the dose, the shorter the incubation period (Kimberlin and Walker, 1978). In mice, the average incubation period was found to increase linearly with logarithmic decrease in dose (McLean and Bostock, 2000). The variations in the incidence of TSE among animals that are naturally exposed to different levels of infection also provide evidence of a dose-response relationship. An increase in the incidence of TSE and a decline in the age at which the disease begins have been reported within affected flocks, and have been attributed to increasing levels of environmental contamination by the infectious agent (Chatelain *et al.*, 1986; Foster and Dickinson, 1989; Sigurdarson, 1991).

2.4.4 Age at exposure

The young age distribution of TSE cases can be explained if (i) the incubation period is shorter in young than in old animals, and (ii) young animals are more susceptible to infection. That younger sheep (less than 18 months) have an increased risk of becoming TSE animals and develop the disease at a rate that is about twice that for older animals (for time < 376) is an interesting observation in this study considering the current age distribution of

natural scrapie cases. This effect remained significant after controlling for other variables. Similar results have been reported by Diaz and colleagues (2005), whereby it was found that animals exposed to infection for the first time early in life appear to be more likely to develop scrapie compared to animals facing infection at older ages. Matthews et al. (2001) suggest that this age effect may be explained by a greater degree of susceptibility of younger compared to older animals, and a relationship also appears to exists between age at infection and age at which clinical signs of scrapie develop (Woolhouse *et al.*, 1998). Results from this present study imply that if young sheep do not develop TSE by the first year of life then it is unlikely that they will develop disease later on. Whether the finding by Diaz and colleagues (2005) may be partly due to intercurrent deaths primarily in older animals is not known. In this present study, the estimate of the HR for older animals is likely to have been biased since the number of intercurrent deaths was significantly greater in older animals. For longer incubation periods/'time till death' (time $\geq$ 376), older animals appeared to have an HR about twice that for younger animals. Whether or not older animals may have developed pre-clinical scrapie and became more susceptible to other disease is not known, although this remains a possibility.

In mice, i/p injections of 10^{-2} inocula of the ME7 strain were found to be 100% lethal in young adults but killed only 80% of neonates, suggesting that neonatal mice probably lack some cell populations required for the initiation of scrapie pathogenesis (Outram *et al.*, 1973). On the other hand, neonatal mice have also been shown to be very susceptible to agent injected by various s/c routes (Outram, 1976). The age of rats at the time of i/c inoculation with TSE-affected material was found to have little effect on the length of the incubation period (Chandler and Fisher, 1963) and this is comparable to findings from this present study. Evidence based on analysis of titration experiments in the murine scrapie model showed that although there was no age effect on probability of infection, young mice appeared to have incubation periods that were, on average, longer than seven days (McLean and Bostock, 2000).

One finding in this study is in contrast to a previous finding where age at challenge was found to have no significant effect on incubation period in sheep (and goats) infected orally with the BSE agent (Foster *et al.*, 2001). However, the sample size used by the authors was small (n = 19) and the majority (85 percent) of sheep challenged were less than 18 months, making it difficult to determine any age effect in light of confounding variables. Huillard d'Aignaux et al. (2002) found no evidence that incubation period of vCJD increases with age

and suggest that the young age of cases of the disease perhaps reflects increased levels of exposure in young individuals rather than age-dependency in the incubation period.

One interesting finding in this present study is that the majority (93%) of young sheep succumbed to TSE prior to 350 days after s/c challenge with a low dose of inoculum, compared to less than 50% of older animals. The s/c route in young sheep appears to be more efficient compared to animals over 18 months, perhaps because of the level of maturation of peripheral nerves. This suggests that if high doses of the agent gain access to the peripheral nervous system, particularly in young animals, then prior amplification in the lymphoid tissue may not be absolutely necessary and the incubation period may be shortened considerably. Where the level of infection is lower, amplification in lymphoid tissue may be required to facilitate TSE pathogenesis, thereby, prolonging incubation periods. It has been found that, following s/c inoculation of neonates (Outram, 1976), 60% of 0-4 day-old mice inoculated with ME7 brain homogenate succumbed to scrapie after short incubation periods (ranging from 150-230 days) and this figure decreased further to 30% in 8 day-old mice and 10% in adult mice.

Various factors contribute to making experimental studies of TSEs difficult. This is largely due to the long incubation period between infection and the development of clinical signs of the disease (resulting in intercurrent deaths as a result of disease other than TSE), and the absence of an ante-mortem diagnostic test (at least during the time at which this experimental study was performed) to detect infected animals. Nevertheless, analyses are relatively more difficult in natural TSEs because of uncontrolled variables such as the time and extent of exposure and the strains of TSE agent encountered throughout the life of the animal. Because of the difficulty in establishing evidence of pre-clinical infection, this made it impossible to verify the initial disease status of the experimental animals. Furthermore, the interval allowed for TSE to develop in the experimental cases was relatively long and this makes it difficult to distinguish experimental cases from cases that may have developed naturally later on (bearing in mind that incubation periods may appear more variable than expected based on genotype/selection line only, and may also reflect the effect of dose and route of inoculation and agent strain, as observed in this present study). For example, in this study, 'age at exposure' was not known for all animals and although animals that developed scrapie at 150 days or less following experimental exposure were excluded from the analyses, the interpretation of the challenge of positive line animals (encoding at least one copy of the VRQ allele) with BSE for instance, may still have been possibly confounded by

the presence of natural scrapie in this line of Cheviots. Foster and others (2001), for example, have shown that incubation times were similar for BSE and Scr8 (a source of natural scrapie) following oral inoculation. Ideally, experiments should be repeated in a scrapie-free environment using scrapie-free sheep from countries (like New Zealand) considered free of the disease.

The diagnosis of TSE was not always confirmed histopathologically, thus introducing criticism of the validity of the outcome of unconfirmed or clinically diagnosed cases. For example, although most sheep exhibit behavioural changes, pruritis, ataxia and weight loss, clinical signs in sheep experimentally infected with SSBP/1 tend to be shorter and weight loss is not a prominent feature (Houston and Gravenor, 2003; Wilson *et al.*, 1950). Nonetheless, even histopathological examination of brain tissue may fail to give positive results in animals incubating disease caused by the SSBP/1 strain (James Foster, personal communication). It is important to note that subclinically infected animals may have similar levels of infectivity as terminally sick animals (Thackray *et al.*, 2002) and, therefore, when determining susceptibility to TSE infection, it is not sufficient to only monitor for clinical signs, but also necessary to assay for PrP^{Sc} and/or prion infectivity (Hill and Collinge, 2003). Differential diagnoses like listeriosis, rabies and pneumonia were never notable in the NPU flock, nor were external parasites. Although toxins are a likely differential, animals were treated prophylactically for several bacterial infections, including clostridial infections. Pregnancy toxaemia can be excluded from the list of differentials since animals were not bred once challenged (James Foster, personal communication). Previous studies investigating the association between flock and management factors and scrapie in the UK have used data that include both confirmed and unconfirmed scrapie flocks (Hoinville *et al.*, 2000a; Hoinville *et al.*, 2000b).

One limitation of this study was the scarcity of genotype information for the sheep used in this study. It has been shown that during an outbreak, the PrP genotypes of affected animals are expected to change over time and "although age-at-onset changed over time, the observed changes were largely, but not exclusively, driven by the time course of the PrP genotypes of cases" (McIntyre et al., 2006). Animals with more resistant genotypes are more likely to live longer (and be present in the flock as older individuals) compared to their younger counterparts. Therefore, whether the age effect observed in this study was driven largely by the time course of PrP genotypes of cases is difficult to determine.

These results show that TSE outcome and incubation period/'time till death' of the disease can be influenced by, in addition to host genetics, the pathogen strain, the dose and route of infection as well as the age at exposure to the agent. The apparent age distribution of the naturally acquired TSEs could perhaps be explained by a shorter incubation period and/or an increased susceptibility to infection in younger animals. Scrapie is similar to vCJD in having both genetic polymorphisms in susceptibility and a long incubation period (Zeidler *et al.*, 1997). However, whether or not findings can be extrapolated to vCJD is questionable. Although some parallels can be drawn with vCJD, direct comparison cannot be made because oral transmission and the species barrier have been shown to lengthen and increase variability in animal experiments (Diringer *et al.*, 1998; Kimberlin and Walker, 1978). Furthermore, other factors not demonstrable by this present study might nevertheless have a potentially important role in the development of TSEs (Diaz *et al.*, 2005; Elsen *et al.*, 1999; Gruner *et al.*, 2004; Healy *et al.*, 2004; O'Rourke *et al.*, 1997), and different host-strain interactions exist under experimental conditions. Although these findings suggest that several interactions exist, the sample size (small relative to the number of interactions incorporated into the model) used in this present study makes it difficult to validate the absolute importance of these interactions.

Why the peak incidence of vCJD (and the other naturally acquired TSEs) is in young individuals is a matter of conjecture in the present state of knowledge but various factors could contribute. This present study raises the possibility of age-related susceptibility, because of yet to be identified biological factors, and may help to explain the controversy that has recently arisen over the importance of various cells, including FDCs (see Chapter 5) and B lymphocytes in the pathogenesis of the TSEs. Furthermore, prediction of the epidemic size of related TSEs, such as vCJD, is difficult in the absence of good understanding of the epidemiology and susceptibility factors for the naturally acquired TSEs. In this study, younger animals appeared to have shorter incubation periods compared to older animals following s/c inoculation of the infectious agent. As mentioned previously, this may be attributed to the greater vulnerability of immature nerves to TSE invasion in the young individual. This observation warrants further investigation of the development of peripheral nerves and their role in determining incubation period of the disease and TSE pathogenesis in sheep. Although this effect was not observed for the oral route, this may be attributed to the small sample size and a difference in the cellular tropism of the respective prion strains, bearing in mind that the BSE agent was used in the majority of oral inoculations as opposed to s/c exposure where the SSBP/1 agent was primarily used.

Nonetheless, oral infection is the most likely route of natural exposure and PPs have been indicated as the most probable sites for the intestinal uptake of prions after oral exposure to the infectious agent (Beekes and McBride, 2000). If the gut immune system plays a role in susceptibility to natural TSE infection it is likely that age will influence susceptibility since the amount of ileal PP tissue decreases with age. In view of the young age distribution of the naturally acquired TSEs including scrapie and vCJD, age-dependent differences in PP development have been investigated, under the assumption that peak development of this lymphoid tissue in adolescent and young adult years may promote TSE replication and consequent development of disease thereby, rendering younger individuals more susceptible to naturally acquired TSEs.

Chapter 3: Quantification of Peyer's patches in Cheviot sheep for future scrapie pathogenesis studies

3.1 Introduction

Susceptibility to vCJD and other TSEs, including scrapie in sheep and BSE in cattle, appears to be age-dependent. This hypothesis is consistent with the observation that sheep first exposed to infection at older ages appear to be less susceptible to scrapie than animals encountering infection for the first time early in life (Diaz et al., 2005; Matthews et al., 2001; Chapter 2 of this thesis). Mathematical models using an estimated age risk function have shown that decreasing age-related susceptibility (with susceptibility peaking in adolescent years) is required to reproduce the characteristics of the age distribution of vCJD cases and that dietary exposure alone could not explain the young age of vCJD cases seen in the UK (Boëlle *et al.*, 2004). Although a relation with puberty may be hypothesised, there is, so far, no clear biological explanation for this peak incidence in young adults.

Oral ingestion is considered to be the main route of natural exposure to TSE agents (Shmakov and Ghosh, 2001). For example, the consumption of BSE-infected beef and beef products is most likely to be responsible for the emergence of vCJD (Bruce *et al.*, 1997), and kuru was likely to be transmitted among the Fore people of Papua, New Guinea by cannibalistic acts at funeral feasts (Huillard d'Aignaux *et al.*, 2002). Following experimental oral infection of mule deer fawns with CWD, PrP^{Sc} was detected first in the lymphoid follicles of Peyer's patches (PPs) and the draining mesenteric lymph nodes 45 days post-inoculation (Sigurdson *et al.*, 1999). When rodents were orally exposed to scrapie, PrP^{Sc} accumulation occurred first in PPs and ganglia of the enteric nervous system (Beekes and McBride, 2000).

In sheep, the ileal PP has been implicated as a likely primary entry site of the scrapie agent followed by spread to more proximal and distal segments of the intestinal tract (Andréoletti *et al.*, 2000). This structure is conspicuous in young animals and can extend up to 2.5 m as a long, continuous aggregate of lymphoid tissue, comprising about 100,000 tightly packed lymphoid follicles (Reynolds *et al.*, 1985; Reynolds, 1987). The total weight (120 grams) of PP tissue is greater than any other single lymphoid organ in the body by 6 weeks after birth (Reynolds and Morris, 1983). What is particularly interesting about the ileal PP is that it undergoes involution, and by about 15 to 18 months of age the organised lymphoid elements of this structure have almost completely regressed. In humans, the number of PPs is greatest

during adolescent (Cornes, 1965) and young adult (van Kruiningen *et al.*, 2002) years and then declines with age. The jejunal PPs by contrast remain intact throughout life in both sheep and humans. An important hypothesis to test was therefore whether this characteristic feature of ileal PP tissue provides a possible biological explanation for the apparent increased susceptibility to TSE infection in young individuals.

It has been suggested that the increased number of PPs in younger animals as well as the increased permeability to macromolecules in the neonatal gut epithelium may contribute to increased susceptibility to oral TSE infection (Shmakov and Ghosh, 2001). If TSE infection starts in the lymphoid follicles of PPs, then it can be speculated that variations in the number and/or surface area of PP tissue in the ileum could explain age-dependent susceptibility to the TSEs and may influence individual susceptibility to disease. For instance, if the follicle-associated epithelium (FAE) is considered as a channel for infection, and assuming that particle uptake capacity is a function of the area of FAE, then reduced gut-associated lymphoid tissue (GALT) may well be associated with decreased incidence of infection (Liebler *et al.*, 1988; Shmakov and Ghosh, 2001).

Because it is so inaccessible to experimental manipulation, the GALT has received considerably less attention than the tonsil or spleen in TSE pathogenesis studies despite the fact that for part of the animal's life the majority of its lymphoid cells reside within this specialized tissue (Reynolds, 1980). This study was undertaken because it was felt that information on the number of lymphoid follicles and surface area of PP tissue in the distal ileum of sheep and their relationship to subject age was essential before a realistic assessment could be made of the potential role of this tissue in determining the age-related incidence of scrapie and the pathogenesis of the disease. This study was not an attempt to repeat previous anatomical work but to obtain data specific to an experimental flock of Cheviot sheep for future scrapie pathogenesis studies. The aim of this study was therefore, to quantify the number of lymphoid follicles and surface area of the ileal PP in different age groups of Cheviot sheep.

3.2 Materials and Methods

3.2.1 Animals

This study was carried out from February, 2003 to October, 2005, using sheep from the NPU Cheviot flock in Edinburgh. The NPU Cheviot flock is a closed flock with endemic natural scrapie and is maintained by the Institute for Animal Health Neuropathogenesis Unit as a source of natural scrapie infections. Demographic information and epidemiological data are available for all sheep (Hunter *et al.*, 1996). Sheep from each of the following age categories were included in the study: 0-3 months, 3-6 months, 6-12 months, 12-18 months and over 18 months (Appendix A, Table A-1). Specimens of the ileum and distal jejunum were obtained from sheep that were either euthanized because of severe arthritis in one or more limbs, died shortly after birth or were culled for flock management reasons. Data including sheep identification number, sex, PrP genotype, date of birth and cause of death were recorded. Investigations were largely restricted to material derived from necropsies carried out within a few hours of death. However, some material (notably from lambs, which were born alive and died during the night) was not examined immediately. Specimens with any clinical or pathological evidence of intestinal disease were excluded from the study. One 7 year-old Suffolk was necropsied at the Easter Bush Veterinary Centre and was used in a preliminary study to help determine the most appropriate method for identification and quantification of PP tissue and lymphoid follicles.

3.2.2 Collection and storage of specimens

Animals were euthanized using sodium pentobarbital (at a dosage of 60 mg/kg, intravenously). A ventral abdominal incision was made, and the caecum exteriorised for orientation. The ileocaecal junction (see Figure 3.1) was identified, and the terminal portion of the distal ileum ligated as close to the junction as possible. The ileum was followed up to 2.5 m proximally and excised, to ensure that the entire ileal PP and a small length of jejunum were included.

Samples of ileal tissue were collected from the caudal end of the distal ileum for histology and ICC (see Chapter 5). Samples for histology were placed in 10% formaldehyde. All other samples were stored in liquid nitrogen at -70°C. The remainder of the specimen was placed in a labelled "zip lock" bag and transported to the laboratory at 4°C.

3.2.3 Visualisation of PPs and lymphoid follicles

The specimens were opened from their caudal ends along the mesenteric borders (to avoid transecting the patches, most of which occur on the antimesenteric aspect) and carefully washed in cold, running water for about 10-15 minutes to help remove mucus and digesta. Although the ileal and jejunal PPs could be seen, LFs were not clearly visible. Preliminary experiments were performed to optimise the technique for counting lymphoid follicles and enhancing the appearance of PPs.

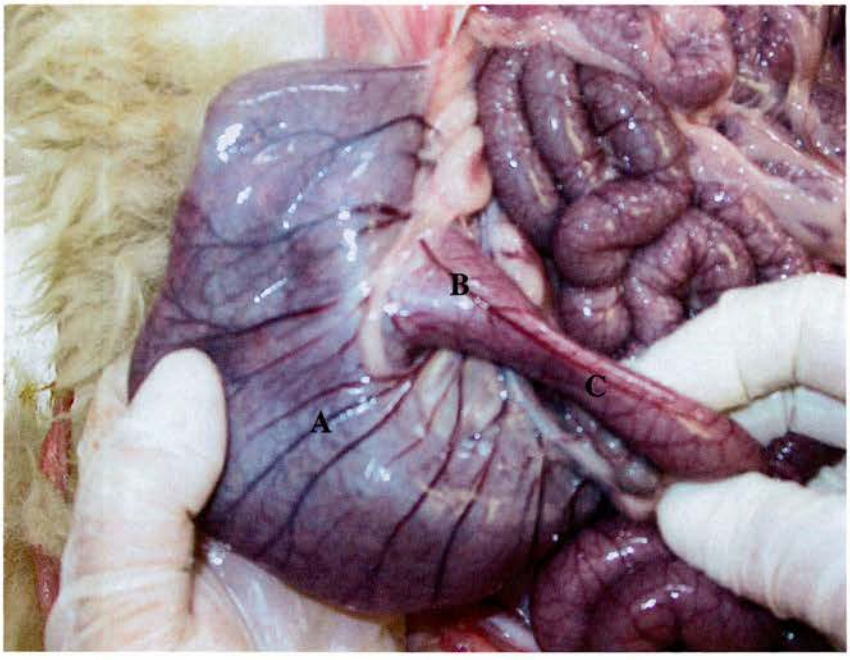

Figure 3.1 Photograph of the caecum **(A)**, ileocaecal junction **(B)** and distal ileum **(C)**

For preliminary studies, the intestine of one 7 year-old Suffolk sheep was divided into several sections (to minimise the number of sheep used). The method used is a modification of Cornes' (1965) method. Segments of intestine were immersed in 2% acetic acid for 24 hours, and then rinsed in cold water (alternatively, specimens may be placed in 50% acetic acid for 2 minutes). Acetic acid is used for precipitation of nucleoproteins (Ronne, 1989), and since lymphoid follicles in PPs are virtually masses of nucleoprotein with little cytoplasm, the lymphoid follicles appear conspicuous. The follicular content of the patches was enhanced by staining with 0.5% methylene blue for 2-5 minutes. To increase the contrast between the dark bluish-black PPs and the lighter bluish-green of the rest of the tissue, specimens were immersed in fresh, dilute (2%) acetic acid for a further 24 hours, followed by a quick wash in water.

3.2.4 Area of PP tissue

After trimming of excess mesenteric fat, the terminal ileum (distal 0.6 m of the ileum) was transilluminated on a horizontal X-ray view box. PP tissue and individual lymphoid follicles were easily visualised using this technique. Digital images were obtained. Image analysis software (Image-Pro Plus®) was then used to calculate the areas of intestine and of PP tissue. The area of PP tissue was recorded as a percentage of the total area of intestinal tissue. Repeatability of results was determined based on paired measurements of percentage of PP tissue for each tissue specimen.

Samples were collected for histology to confirm that the patches were in fact collections of lymphoid follicles, and that this method succeeded in staining the lymphoid follicles and no other structure of the same size and shape. Jejunal PPs were collected for comparative purposes.

3.2.5 Quantification of lymphoid follicles

In young lambs (less than 6 months old), the serosal surface of the intestine was removed to allow for easy quantification of lymphoid follicles. This was done by carefully slitting the serosal tissue at right angles to the long axis of the intestine and stripping away the serosa with a pair of forceps. With practice, it is quite easy to remove the serosal membrane. To determine the number of lymphoid follicles, the stained intestine was placed between two glass slides, the upper of which was etched in square centimetres. Individual lymphoid follicles appeared as bright blue spots against a faintly blue background when viewed on the X-ray box. The number of lymphoid follicles in 6 different sections along the length of the terminal ileum was counted by naked eye, starting at 5 cm from its caudal end and selecting 4 cm^2 sections at every 10 cm thereon, proximally. In older sheep (over 6 months), lymphoid follicles were smaller in size and more difficult to visualize. In these animals, follicles were counted from the mucosal surface of the intestine using a dissecting microscope (magnification of X4). Results were recorded as the average number of lymphoid follicles per cm^2 of ileum. Repeatability of results was determined based on two separate counts for each of the 6 tissue sections.

3.2.6 Techniques

Throughout the investigation, every attempt was made to obtain tissues within one or two hours of death followed by immediate refrigeration. Samples that were obtained several

hours after death were friable and difficult to handle. They stained poorly and failed to demonstrate PPs in autolytic areas of the intestine.

To optimise results, the following points were noted during the course of this study. Fresh intestinal tissue (obtained at least within one or two hours of death) should be used and washed in cold, running water. Water should not be forced through the lumen in an attempt to flush out digesta since there is the risk of damaging lymphoid follicles and disrupting villi. Formalin was not appropriate for this study since it resulted in shrinkage and discolouration of the tissue making it difficult to visualise patches altogether (not shown). Therefore, for a more detailed gross morphologic study, the specimen should be placed in dilute acetic acid and not formalin.

3.2.7 Statistics

The repeatability of a method evaluates the extent to which the same observer obtains the same results under identical circumstances. In this study, repeatability of the two methods used was determined based on two separate estimates for each specimen. To assess repeatability of the methods used, the difference between the pair of measurements for each individual was calculated and the limits of agreement (that is, the limits within which most of the differences lie) calculated (Petrie and Watson, 2001). Assuming the differences follow a normal distribution, the limits are calculated as:

$d \pm 2s_{diff}$, where

d is the mean of the differences, and
s_{diff} is the standard deviation of the differences

To check that the relationship between the differences and the magnitude of the measurement is constant, the difference in the two measurements in a pair against their average is plotted. A random scatter of points around the line of zero difference with no funnel effect as the mean of the two measurements increases indicates a constant relationship. Lines that denote the upper and lower difference values that enclose 95% of the points are added to the plot. Hence the name limits of agreement plot (or Bland-Altman plot) (Bland and Altman, 1986).

The Spearman rank correlation coefficient (Petrie and Watson, 2001) was used as a measure of association between the anatomical data and the corresponding age of an individual.

3.3 Results

A total of 41 tissue specimens were examined. Eight of these specimens were excluded from the study because of evidence of parasitism, or because lambs had died during the night and the intestines had undergone post-mortem changes.

3.3.1 Area of PP tissue and lymphoid follicle density

The ileal PP was continuous in younger animals (0-6 months) and was found to extend up to 1.39 m proximally (Appendix A, Table A-1). In old animals, the ileal PP was found to have a "moth-eaten" appearance when stained with methylene blue. This is consistent with involution and associated loss of follicles from PP tissue. PP tissue was present largely on the anti-mesenteric aspect of the small intestine. After puberty (about 12 months), a drastic decrease in the area of ileal PP tissue was observed (Figure 3.2). The area of ileal PP tissue ranged from a mean of 49.8 to 60.3 % in 0-3 month-old lambs to 0 to 7.0 % in the oldest animals (18 months and over). The percentage of ileal PP tissue was negatively correlated with age of the animal (r_s = -0.879; n=33, P<0.0001).

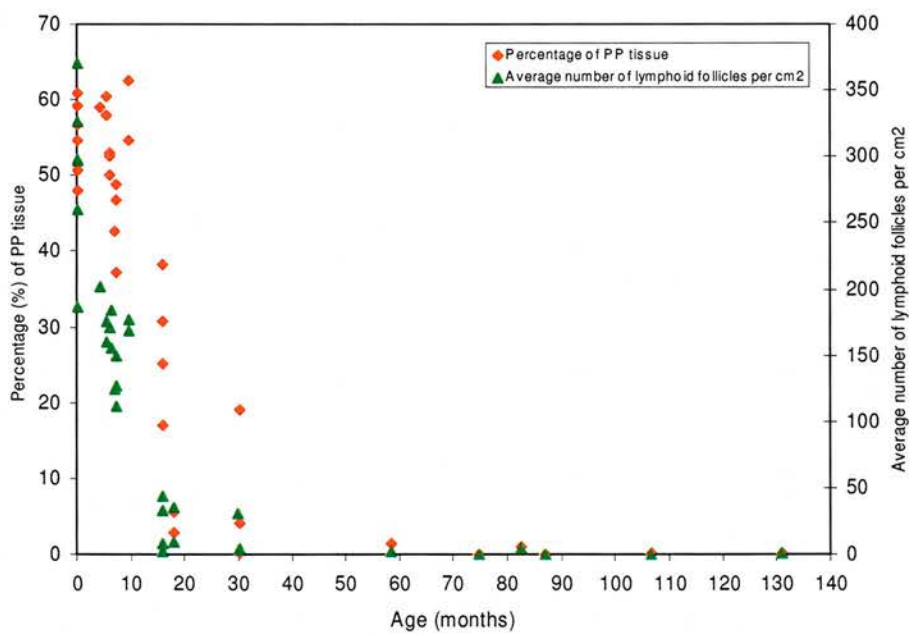

Figure 3.2 Percentage of PP tissue and the average number of lymphoid follicles per cm^2 in sheep of various ages (n=33).

Lymphoid follicle number is represented as the average number of lymphoid follicles per cm^2 of terminal ileum as shown in Figure 3.2. Lymphoid follicles were most numerous near the ileocaecal junction. Compared to the area of ileal PP tissue, a similar drastic decrease in lymphoid follicle density was observed after puberty (Figure 3.2). There was some variation in the number of follicles between individual animals in each age group; however, variation between the respective age groups was striking. Lymphoid follicle density ranged from a mean of 224 to 355 in 0-3 month-old lambs to 0 to 17 in the oldest animals (18 months and over). Lymphoid follicle density was negatively correlated with age of the animal (r_s = -0.943; n=33, P<0.0001). Area of ileal PP tissue and lymphoid follicle density were found to be highly concordant (r_s = 0.899, n=33, P<0.0001). The partial correlation coefficient for the two variables after adjusting for the effect of age was 0.440, n=33, P=0.01.

3.3.2 Follicular pattern

The macroscopic appearance of lymphoid follicles in young lambs (0-6 months) was characteristic (Figure 3.3). Once the serosal surface of the intestine was removed, lymphoid follicles appeared as tiny, raised white plaques (of about 0.5 mm in diameter) (Figure 3.3A). The follicles were numerous and appeared to be evenly distributed throughout the entire ileal PP. When the specimen was transilluminated on an X-ray box, the follicles appeared as dark blue nodules (Figure 3.3B), indicating uptake of methylene blue stain. Placing the specimen in water (or 2% acetic acid) appeared to remove the colour from surrounding tissues more quickly than from the follicles. Lymphoid follicles were less obvious from the serosal surface and far less numerous in older (over 18 months) animals. Follicles in these animals were more easily observed on the mucosal surface as tiny, light blue nodules present among the villi of the intestine (not shown).

Lymphoid follicles were characterised microscopically as well defined focal cellular aggregates of lymphoid cells (lymphocytes and lymphoblasts) with variable numbers of associated tingible body macrophages. A morphologic difference in lymphoid follicles between young and older animals was apparent. In young (0-6 month-old) animals, the follicles were more numerous and larger, and tended to extend throughout the entire lamina propria of the intestine (Figure 3.4A). Coccidia were found to be present in association with the mucosal epithelium in some samples; these specimens were excluded from the study. Few lymphoid follicles were found in animals aged 18 months and older (Figure 3.4C).

Lymphoid follicles in these animals were less extensive compared to those seen in younger animals (Figure 3.4A and B). Figure 3.4C shows one lymphoid follicle from a section of ileal tissue in a 30 month-old ewe. The lymphoid follicle has undergone involution and may not be visible macroscopically from the mucosal surface.

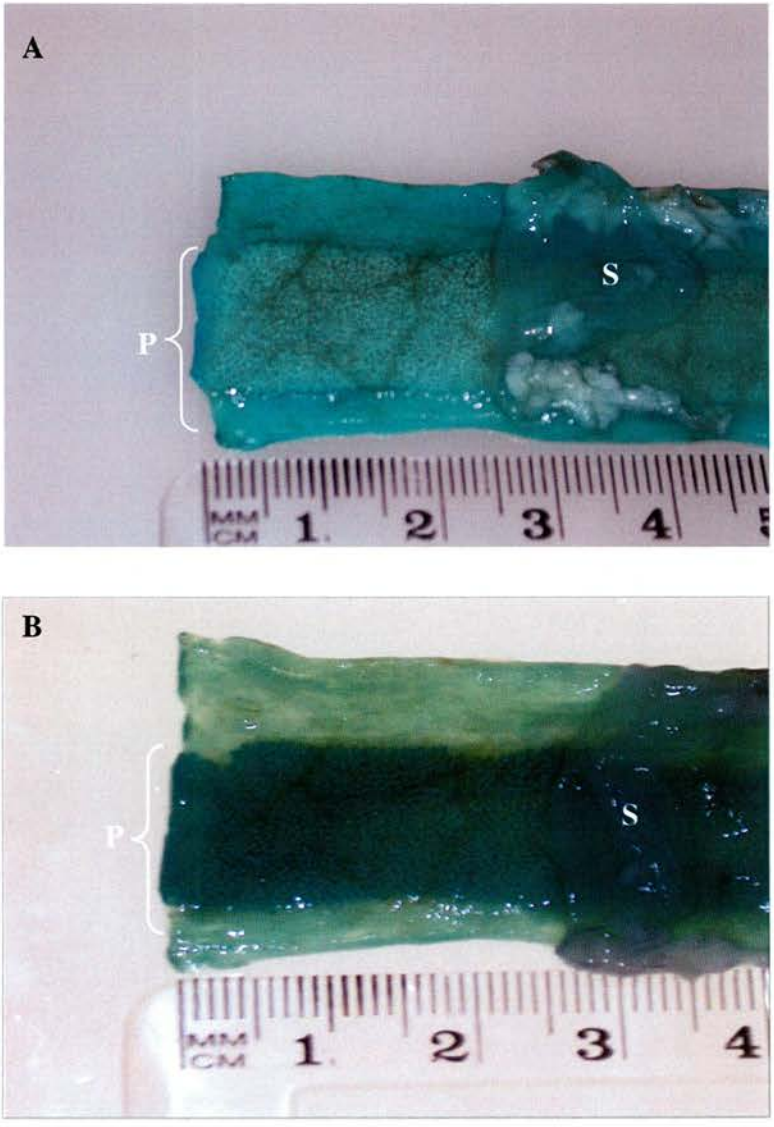

Figure 3.3 Lymphoid follicles in a 4-month old lamb. The serosal membrane (S) was partly stripped to reveal the underlying lymphoid follicles. **A**. No transillumination has been used, and the lymphoid follicles appear as tiny, white nodules. **B**. The tissue section has been transilluminated and the follicles appear as dark, greenish-blue nodules. P= ileal Peyer's patch.

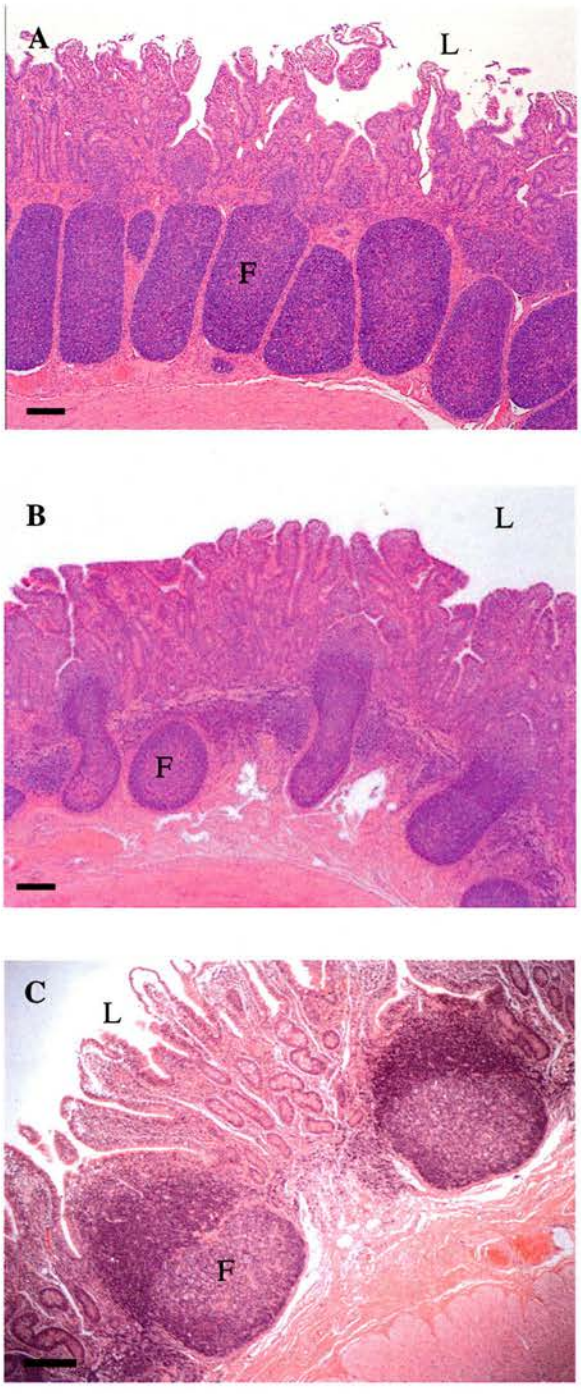

Figure 3.4 Comparison of PP lymphoid follicles in the ileum of NPU Cheviot sheep at: **A**. 3 months; **B**. 7 months, and **C**. 30 months. Lymphoid follicles undergo involution and are fewer in number with increasing age. F=lymphoid follicle; L=intestinal lumen. Bar = 200 μm.

3.3.3 Statistics

3.3.3.1 Measuring Agreement

The Bland and Altman plot was used to assess the repeatability of each method (that is, using lymphoid follicle counts and area of PP tissue) by comparing repeated measurements for each method on 33 sheep. Plots were used to determine whether the variability or precision of each method is related to the size of the characteristic (lymphoid follicle counts or area of PP tissue) being measured.

Repeatability of the methods used for quantifying area of PP tissue and number of lymphoid follicles was satisfactory and paired measurements per individual animal can be used with reasonable confidence. Limits of agreement (Figure 3.5) were obtained in order to determine if the relationship between the differences and the magnitude of the measurement (whether large or small) is constant. With the exception of a small cluster of points at the extreme left of the line of zero differences (representing sheep with very few or no lymphoid follicles or PP tissue), the relationship between the differences and the magnitude of the measurement is more or less constant for both methods as can be seen from the random scatter of points around the line of zero differences (Figure 3.5). We expect 95% of differences to lie within the limits -143 to 181 (19 ± 162) and -1.15 to 1.49 (0.17 ± 1.32) for lymphoid follicle density and area of PP tissue, respectively.

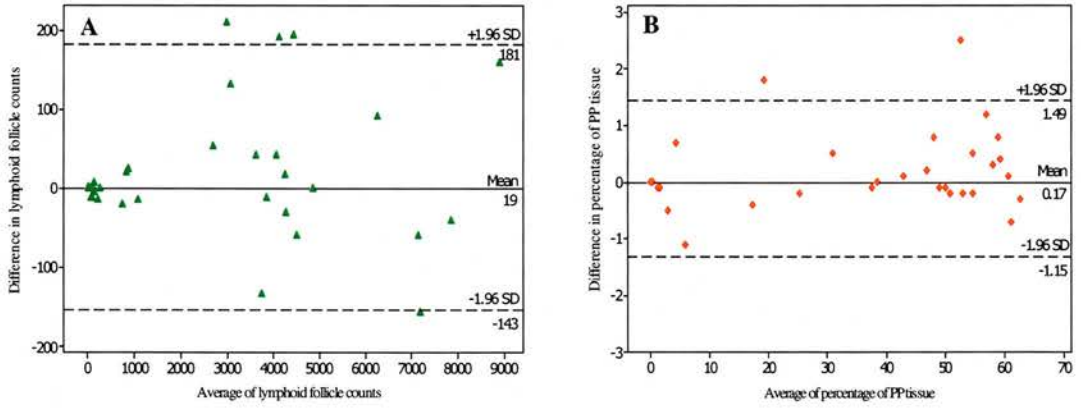

Figure 3.5 Bland and Altman plots for assessing the repeatability of methods used for quantifying lymphoid follicles and area of PP tissue. **A.** The difference between two lymphoid follicle counts plotted against their average, and **B.** The difference between two measurements of percentage of PP tissue plotted against their average.

3.4 Discussion

Young sheep have distinctly more follicles and PP tissue than adults. Although there was some individual variation in the measures (surface area of PP tissue and number of follicles), the general tendency was a rapid falling off in follicle number and patch area immediately after 12 months of age. For purposes of comparison between different cases, the average number of follicles per cm^2 and average PP surface area are obviously better measures than the total number of follicles and total PP surface area because of the different dimensions of the ileum in different sized individuals. Direct comparisons with previous studies proved difficult primarily because of different aged animals and measures used for quantifying PP tissue and follicular content. One study (Reynolds and Morris, 1983) showed that the weight of ileocaecal PP tissue and the associated number of follicles were greatest for 9 week-old lambs. However, in this study, cases ranged from 120 days gestation to 9 weeks post-partum and did not always account for differences in intestinal length in different sized individuals. Also, the breed of sheep used in the study was not stated.

Several factors may influence the number of PPs in the intestine, for example, diet (and hence the antigenic load in the intestine), the frequency of food consumption as well as the time taken between ingestion and evacuation may well be important factors (Poskitt *et al.*, 1984). Whether or not variations in PP tissue area and lymphoid follicle number arise depending on exposure to amount and type of antigen, or whether the development of lymphoid follicles is embryologically pre-determined for the breed or the individual animal (Liebler *et al.*, 1988) remains unknown.

In sheep, natural scrapie infection occurs most likely by the oral route via infection of PPs followed by replication in the GALT (Andréoletti *et al.*, 2000). Replication in the intestine and lymphoid organs usually occurs for many months, even years, before reaching the CNS via the blood or nerve fibres innervating lymphoid tissues (Andréoletti *et al.*, 2000). FDCs have been implicated as the cells of the lymphoid system that sustain replication of the TSE agent in laboratory rodents (Brown *et al.*, 1999; McBride *et al.*, 1992). Studies in sheep have shown the presence of PrP^{Sc}, which is strongly associated with disease (Bolton *et al.*, 1982), in cells of the FAE (Heggebø *et al.*, 2000), and this is consistent with PrP uptake from the gut lumen occurring across this specialised intestinal epithelium. This transport function is thought to be mediated by M cells, which as shown in one study, are able to mediate transepithelial transport of prions (Heppner *et al.*, 2001). Transcytosis of pathological agents across the FAE has been implicated in several diseases including *Yersinia* infections,

salmonellosis and paratuberculosis (Gleason and Patterson, 1982; Landsverk *et al.*, 1990; Momotani *et al.*, 1988; Wolf and Bye, 1984) which are all known to originate in PPs.

Natural infection with the scrapie agent is thought to occur mainly in young sheep less than 9 months old (Hourrigan *et al.*, 1979). During this period of high susceptibility to infection, the ileal PP serves as the main GALT comprising an extensive area of FDCs and specialised FAE actively engaged in the uptake and transcytosis of macromolecules from the gut lumen (Heggebø *et al.*, 2000). Involution of the ileal PP at puberty and the accompanying drastic reduction in the number of follicles (and FDCs as well as FAE) may well contribute to reduced susceptibility to scrapie infection observed in older animals (Diaz *et al.*, 2005; Hourrigan *et al.*, 1979) and may influence individual susceptibility to disease. A number of other factors including PrP genotype, strain of agent and environmental factors such as artificial or natural rearing (Elsen *et al.*, 1999; Hunter *et al.*, 1996) have been linked with susceptibility to scrapie. The incubation period and pathogenesis of scrapie varies according to the interaction of a number of these factors (as demonstrated in Chapter 2). Hence, it is becoming clear that there is great heterogeneity in the control of susceptibility to scrapie, and the association made here suggests that the amount and distribution of ileal PP tissue may represent an additional contributory factor.

One study showed that sheep parasitized with *Teladorsagia circumcincta* were at a greater risk of becoming infected with the scrapie agent. However, results were inconclusive because of improper controls, and the possibility that subclinical scrapie may have already been present in the flock (Elsen *et al.*, 1999). Specifically, it has been suggested that infection with *Teladorsagia circumcincta* may be a cofactor of scrapie infection, facilitating penetration of the infectious agent through lesions due to larvae in the gastrointestinal tract (Laplanche *et al.*, 1996). It has been hypothesised that nematodes may serve as vectors of the disease, but this hypothesis has, so far, been tested with no success (Fitzsimmons and Pattison, 1968). Another possibility is that PP numbers are perhaps increased in parasitic infections and bearing in mind that these lymphoid structures most likely act as a primary replication site and reservoir of the scrapie agent (Andréoletti *et al.*, 2000) results in parasitized individuals becoming more susceptible to scrapie infection. Further studies of ileal PP tissue in different age groups of sheep with disease conditions are therefore needed in order to obtain more information about the functional significance of this specialized gut tissue and its role in scrapie pathogenesis.

Chapter 4: Comparative evidence for a link between PP development and susceptibility to TSEs

4.1 Introduction

The incidence of natural cases of TSEs or prion diseases is related to age: scrapie incidence in sheep typically peaks between 2 and 3 years of age (Detweiler and Baylis, 2003), BSE incidence in cattle peaks at around 5 to 7 years of age (Defra, 2004); and vCJD incidence in humans peaks at 25 to 30 years (Will, 2003). Age-related patterns in incidence will reflect the incubation period of the disease (typically long relative to host life expectancy), the magnitude of the risk of infection and any age dependency in the risk of infection. Analyses of epidemiological data for scrapie (Matthews *et al.*, 2001), BSE (Ferguson *et al.*, 1997) and vCJD (Boëlle *et al.*, 2004) have suggested that there is significant age dependency in the risk of infection for all these TSEs. Available evidence suggests that these patterns cannot be fully accounted for by changes in exposure, in which case changes in susceptibility must also play a role. However, to date, there has been no indication of why susceptibility might change with age.

Age dependency in the risk of infection by TSEs will reflect any age dependency in exposure to infection and/or in susceptibility to infection for a given level of exposure. Both of these are likely to be linked to the route of transmission. Although other transmission routes may exist (see below), oral exposure appears to be the most important route of transmission for natural TSE infections in sheep, cattle, deer and mink and for vCJD and kuru in humans (Detweiler and Baylis, 2003; Heggebø *et al.*, 2000; Sigurdson *et al.*, 1999; Terry *et al.*, 2003; Will, 2003). There is evidence for the involvement of Peyer's patches (PPs), part of the gut-associated lymphoid tissue (GALT), in orally transmitted TSE infection. Experimental studies in cattle have demonstrated staining for PrP^{Sc} (the abnormal prion protein) in PP follicles in the distal ileum throughout much of the course of the disease following oral exposure to the BSE agent (Terry *et al.*, 2003). In sheep, oral infection with scrapie is thought to occur mainly via the ileal PP, followed by replication in GALT (Heggebø *et al.*, 2000). In mule deer fawns, lymphoid follicles of PPs have been shown to accumulate PrP^{Sc} within a few weeks following oral exposure to CWD (Sigurdson *et al.*, 1999). After oral infection of nonhuman primates with BSE-infected material, PrP^{Sc} is initially detected in PPs (Bons *et al.*, 1999). In experimental infections, mice deficient in both tumour necrosis factor (TNF) and lymphotoxin or in lymphocytes, in which PPs are decreased in number, are highly resistant to oral challenge and their intestines are virtually devoid of infectivity at all times

post-challenge (Prinz *et al.*, 2003). These facts collectively suggest a key role for PPs in the infection dynamics of a range of TSEs.

Early presence of PrP^{Sc} in mouse PPs after oral exposure to scrapie {Beekes and McBride 2000) has indicated these structures as being the most probable sites for the intestinal uptake of the TSE agent. Various cell types present in this lymphoid tissue have been implicated as important elements in the uptake and propagation of the infectious agent. PrP^{Sc} staining in the follicular dendritic cells (FDCs) of patients with vCJD (Hill *et al.*, 1999) and of sheep naturally infected with scrapie (van Keulen *et al.*, 1996), as well as staining associated with the luminal border of cells in the follicle-associated epithelium (FAE) of sheep suggest uptake of the TSE agent from the intestinal lumen to the underlying lymphoid tissue (Heggebø *et al.*, 2000). Although important functional differences exist between PP in sheep ileum and those in the duodenum and jejunum, the FAE overlying jejunal and ileal PPs has an efficient mechanism for the transcytosis of luminal material (Griebel and Hein, 1996; Lie *et al.*, 2005), including prion proteins, to the underlying lymphoid tissue.

The development of GALT is known to be related to age. In young sheep, cattle and humans, ileal PPs are the major component of GALT possessing an extensive bed of FDCs and FAE. The involution of ileal PPs occurs at around puberty in sheep, cattle and humans (Carlens, 1928; Cornes, 1965; Reynolds and Morris, 1983). However, the age-related changes in PP development are not identical across these three species, providing an opportunity for a comparative study. Our hypothesis is that although the relationships between PP development and age and between susceptibility to TSE infection and age differ in sheep, cattle and humans, there should still be a correlation between PP development and susceptibility for each species.

4.2 Methods

4.2.1 Anatomical studies

Specimens of ileum were collected from 33 sheep of different ages (0-1 year, 1-2 years and >2 years) from a flock of Cheviot sheep maintained by the Institute for Animal Health NPU (Hunter *et al.*, 1996). The study was limited to animals with no clinical or pathological evidence of intestinal disease. Specimens were obtained from sheep that were either euthanised because of severe arthritis in one or more limbs, died shortly after birth or were culled for flock management reasons. The specimens were opened along their mesenteric borders, and rinsed in cold water. PP tissue and lymphoid follicles were visualised by

immersing the intestines in 2% acetic acid for 24 hours, and the follicular content of the patches enhanced by staining with 0.5% methylene blue for 2-5 minutes. PP tissue and lymphoid follicles were easily visualised using this technique.

The terminal ileum (distal 0.6 m of the ileum) was transilluminated on a horizontal X-ray view box and digital images were obtained. Image analysis software (Image-Pro Plus®) was then used to calculate the areas of intestine and of PP tissue. The area of PP tissue was recorded as a percentage of the total area of intestinal tissue.

To determine the number of lymphoid follicles, the stained intestine was placed between two glass slides, the upper of which was etched in square centimetres. Individual lymphoid follicles appeared as bright blue spots against a faintly blue background when viewed on the X-ray box. The number of lymphoid follicles in 6 different sections along the length of the terminal ileum was counted by naked eye, starting at 5 cm from its caudal end and selecting 4 cm^2 sections at every 10 cm thereon, proximally. Results were recorded as the average number of lymphoid follicles per cm^2 of ileum.

Results are described in terms of area of PP tissue and lymphoid follicle density in the sheep ileum; analyses indicate that these two measures are closely correlated (r_s=0.899, n=33, P<0 .001). PP data for cattle and humans were obtained from earlier studies (Carlens, 1928; Cornes, 1965). These studies used different measures to quantify PP tissue from those we obtained here for sheep. The cattle data (Carlens, 1928) refer to weight of PP tissue in the small intestine of 94 German beef cattle. The human data (Cornes, 1965) refer to number of PPs in the normal small intestine of 46 individuals between 0 and 40 years of age. The study was limited to necropsies performed within a few hours of death, and to patients with no clinical history or pathological evidence of gastrointestinal tract disease. A second, smaller study of human PPs indicates that, in humans, number of PPs and area of PP tissue in the distal ileum were correlated across age classes (r=0.415, n=55, P<0.01) (van Kruiningen *et al.*, 2002). As far as I am aware, there are no other quantitative data on PP development with respect to age available for these species but, where direct comparisons are possible, it appears that the different measures reflect the same underlying relationship with age.

4.2.2 Scrapie incidence data

The NPU Cheviot flock, a closed flock maintained explicitly as a source of natural scrapie infections, has been comprehensively documented and demographic information and

epidemiological data on all sheep are available (Hunter *et al.*, 1996). In this study, analyses were based on data obtained from an outbreak of scrapie, which spanned the years 1985 to 1994 affecting cohorts born between 1983 and 1992. This represents a total of 1,473 sheep of which 34 developed clinical scrapie. In this flock, scrapie occurs in two PrP genotypes, VRQ/VRQ and VRQ/ARQ (Hunter *et al.*, 1996). (There is no evidence that PrP genotype influences PP development). Further details of the outbreak are given elsewhere (Hunter *et al.*, 1996).

4.2.3 Age-susceptibility function for sheep

The method follows that of Boëlle et al. (Boëlle *et al.*, 2004) used to derive the age risk function for vCJD. The occurrence of cases in genotype G sheep is modelled by a Poisson process in the (age, time) plane with intensity $\pi_G(a,t)$ given by:

$$\pi_G(a,t) = \beta_G r_G S(a) \int_0^a \exp\left[-\int_0^{a'} \lambda(u, t-a+u)du \right] \lambda(a', t-a+a') h_G(a-a')da'$$

where β_G is the birth rate and r_G is the relative susceptibility of genotype G individuals, $S(a)$ is the probability of survival (in the absence of scrapie) until age a, h_G is the probability density function for the incubation period for genotype G individuals, $\lambda(a,t)$ is the per capita rate of infection for individuals of age a at time t. The expression sums the contribution to the incidence of infection at age a and time t from animals infected when at age a', taking into account the fact that the number of animals available at age a' to become infected is reduced by those already infected at age u. The low incidence of scrapie in this flock (Matthews *et al.*, 2001) permits modelling of the age and timing of cases as a Poisson process because the course of the outbreak does not significantly impact on the demography of the susceptible sheep.

The survivorship function $S(a)$ is a Weibull function with mean age of death of 2.99 years (Matthews *et al.*, 2001). The incubation period distribution is a gamma distribution with a mean of 1.9 years (Matthews *et al.*, 2001). The birth rate β_G is selected to give the average numbers of sheep of different genotypes born per year. The per capita rate of infection, $\lambda(a,t)$ has two parts: a time dependent component $g(t)$ which is assumed here to be proportional to an exponential function fitted to the incidence of infection; and an age-dependent component f(a) which represents the relative susceptibilities of different age classes:

$$f(a) = \begin{cases} f_1 & \text{for} \quad 0 \le a < 1 \\ f_2 & \text{for} \quad 1 \le a < 2 \\ f_3 & \text{for} \quad 2 \le a < 9 \end{cases}$$

where the maximum value taken by f_1 f_2 or f_3 is equal to 1. Standard theory on point processes (Cox and Isham, 1980), gives the log-likelihood of the observed age-of-case data to be:

$$\sum_{i}^{N_{cases}} \log(\pi_{G_i}(a_i, t_i)) - \sum_{G} \int \int \pi_G(a,t) da dt$$

The subscript i denotes actual case data; deaths are known to occur at age a_i and a time t_i after the start of the outbreak. Maximum likelihood methods were used to estimate the constant of proportionality, which determines the magnitude of the per capita rate of infection and the age-dependent susceptibility function as defined by f_1, f_2, and f_3. This was done for (i) the 34 cases over the 10 year period assuming no differences between genotypes, and (ii) for the 28 genotyped cases allowing the 8 VRQ/ARQ cases to have either a lower susceptibility to infection or (iii) a longer incubation period than the 20 VRQ/VRQ cases. We found that models (ii) and (iii) produced a significant improvement in fit at the 95% level over model (i), but that the shape of the age-dependent susceptibility function was robust to the choice of model. Results are shown for model (ii).

For cattle, estimates of risk of BSE infection were made from n=158,550 BSE cases in British cattle and were calculated from the cumulative distribution function, defined by Ferguson *et al.* (1997), corresponding to the age-exposure/susceptibility curve (fitted using maximum likelihood methods).

For humans, estimates of risk of vCJD infection were obtained from a previous study that comprised n=129 vCJD cases in British people, and were fitted using maximum likelihood methods by Boëlle *et al.* (2004).

4.2.4 Concordance between susceptibility data and anatomical data

For each combination of anatomical data and risk of infection estimates we calculated the Spearman's rank correlation coefficient, r_s, between the value of the available measure of PP development (area, weight or number) and the risk of infection for an individual of the

corresponding age. Sample sizes were n=33, n=94 and n=46 for sheep, cattle and humans, respectively. Correlation coefficients were calculated using S-PLUS 2000 for Windows.

4.3 Results

For sheep, cattle and humans alike, a strong correlation was found between risk of TSE infection and the development of lymphoid tissue in the gut which can explain both the relationships between age and disease incidence within species and differences in this relationship between species.

For sheep there is a marked fall in both the surface area of ileal PP tissue and lymphoid follicle density between approximately 12 and 24 months old, and both measures remain very low throughout adulthood (Figure 4.1 and 4.2A). Analysis of data on the incidence of natural scrapie over a 10 year period in the sheep flock providing the anatomical data indicates that the risk of infection is highest in the first year of life and is lowest in sheep >2 years old (Figure 4.2A). The two distributions peak in the same age class and are highly concordant (see Methods): surface area of ileal PP tissue vs risk of infection, r_s=0.887 (n=33, P<0 .001); lymphoid follicle density vs risk of infection, r_s=0.882 (n=33, P<0.001).

For cattle, previous work (Carlens, 1928) has shown that the weight of PP tissue in the small intestine increases in the first year of life, peaks at 12-18 months old, declines thereafter, and is low throughout adulthood (Figure 4.2B). Available estimates of age-related risk of infection of the British cattle population with BSE up to 1996 (given a mean incubation period of 5 years; published estimates range from 4.5 to 5.5 years (Arnold and Wilesmith, 2004)) indicate that the risk is initially low, peaks at about 12 months, and declines rapidly thereafter (Figure 4.2B). Again, the two distributions peak at similar ages and are concordant: r_s=0.693 (n=94, P<0.001).

For humans, previous work (Cornes, 1965) has shown that the number of PPs in the small intestine increases during childhood, peaks at 10-15 years old, and declines thereafter, although the PPs persist throughout adulthood (Figure 4.2C). Recent estimates of age-related risk of infection of the British human population to vCJD (Boëlle *et al.*, 2004) indicate that the risk is initially low, peaks between 5 and 20 years, and declines thereafter (Figure 4.2C). Here too, the two age-related patterns are concordant: r_s=0.38 (n=46, P=0 .008). The same study (Boëlle *et al.*, 2004) provides estimates of age-related susceptibility having allowed for changes in putative exposure associated with consumption of bovine carcass meat (see

below). This is also concordant with the number of PPs: r_s=0.360 (n=46, P=0.014). Importantly, these correlations occur despite the markedly different patterns of age-related development of GALT in humans as compared with sheep and cattle.

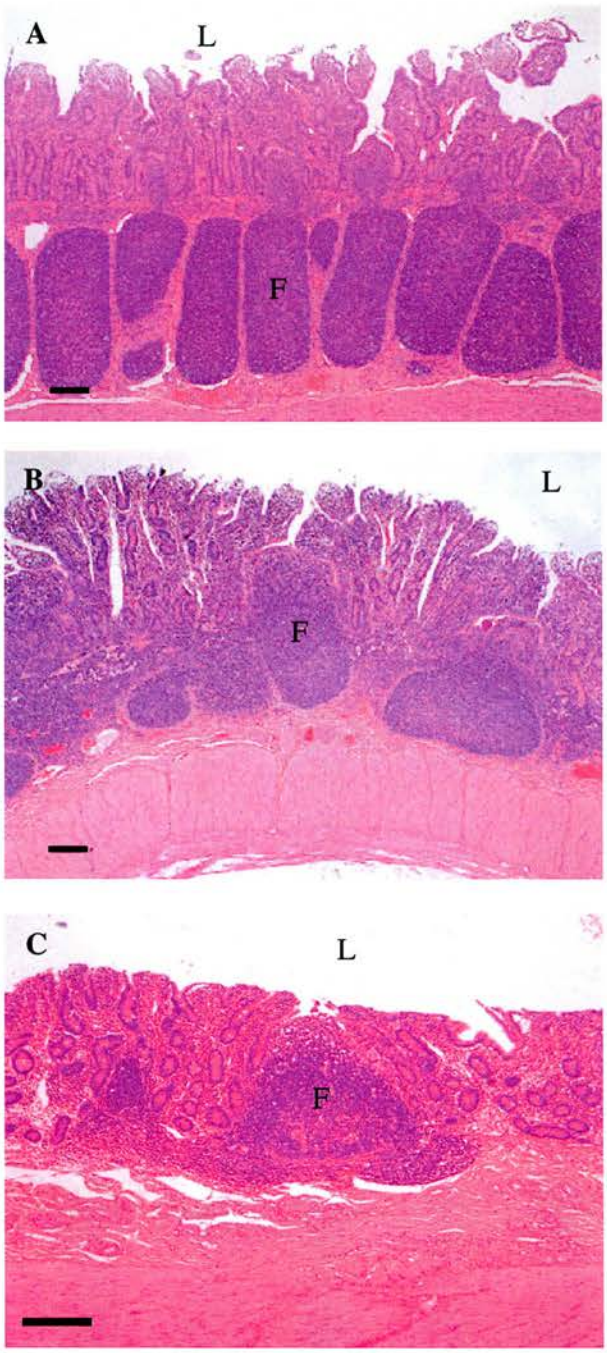

Figure 4.1 Comparison of PP lymphoid follicles in the ileum of NPU Cheviot sheep at: **A**. 4 months; **B**. 15 months, and **C**. 6 years, using haematoxylin and eosin staining. Lymphoid follicles (F) undergo involution and are fewer in number with increasing age. L=intestinal lumen. Bar = 200 μm.

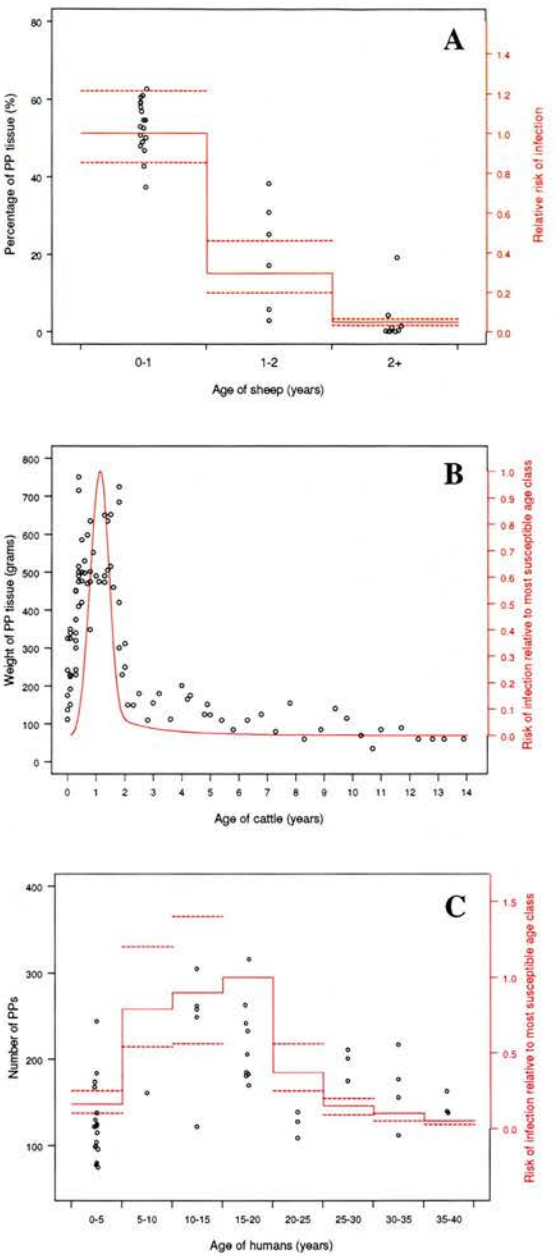

Figure 4.2 Comparison of age-related changes in PP development and estimated risk of TSE infection relative to the most susceptible age class, for sheep, cattle and humans. **A**. PP areas for n=33 Cheviot sheep of mixed genotypes in 3 age classes (left hand axis, open circles), compared with estimates of risk of scrapie infection relative to the most susceptible age class (solid line) (±50 percentiles-dashed lines) from field data on n=34 cases in mixed PrP genotype (VRQ/VRQ and VRQ/ARQ) Cheviot sheep (see Methods) in the same age classes (right hand axis). **B**. PP tissue weight against age for n=94 cattle (open points, data from Carlens, 1928), compared with estimates of risk of BSE infection relative to the most susceptible age class (solid line) as a function of age made from n=158,550 BSE cases in British cattle (taken from Ferguson *et al.*, 1997). **C**. Numbers of PPs in the small intestine in 8 age classes of humans (open circles, data taken from Cornes, 1965), compared with estimates of risk of vCJD infection relative to the most susceptible age class (solid line, ±50 percentiles-dashed lines) from n=129 vCJD cases in British people for the same age classes (redrawn from Boëlle *et al.*, 2004).

4.4 Discussion

Results show that, whilst both age-related changes in the development of PP tissue and estimated risks of TSE infection differ between sheep, cattle and humans, in each case the two are associated. However, these results do not distinguish effects of age-related changes in exposure to TSE infection from age-related changes in susceptibility. To make this distinction we need to consider how oral exposure to TSE infection might change with age for each species.

For BSE in cattle, epidemiological studies have implicated meat and bone meal (MBM) containing recycled infected cattle tissues (Wilesmith *et al.*, 1988). MBM used to be incorporated as a protein source in concentrated feedstuffs and fed to both calves and adult cattle. However, there is no clear correlation with the estimated age-infection function (Figure 4.2B): almost all calves were exposed to MBM by 6 weeks of age; exposure then fluctuated up to 24 months old but, especially for dairy cows, rose again in adulthood (Anderson *et al.*, 1996; Wilesmith *et al.*, 1997). This route of BSE transmission is thought now to have been eliminated by feed production regulations introduced in 1988 and 1996.

For vCJD in humans, the most likely vehicle for exposure is food products containing BSE-contaminated cattle tissues (Cooper and Bird, 2002). Humans consume solid foods from 4-6 months of age with average consumption of bovine carcass meat peaking during childhood and tending to fall thereafter (see Figure 3 in Boëlle *et al.*, 2004). This route of transmission is thought now to have been eliminated by food production regulations introduced in the UK in 1996. Here, putative exposure is more closely aligned with PP development (Boëlle *et al.*, 2004) but, as reported above, when age-related exposure is taken into account, there remains an association between PP development and estimated susceptibility.

For scrapie in sheep, the vehicle(s) of oral exposure is (are) less well understood, but are likely to include grazing on pasture contaminated with scrapie, possibly by infected foetal membranes (Pattison *et al.*, 1972). Lambs typically begin to graze at 6-14 weeks and continue to do so throughout their lives. Exposure by this route would not be correlated with the estimated age-infection function (Figure 4.2A).

The importance of other transmission routes is less clear. Transmission from mother to offspring in utero or via breast milk (self-evidently age-dependent) is thought to play a minor role, if any: currently available estimates of the fraction of cases due to maternal

transmission are 0-8% for scrapie in sheep (Matthews *et al.*, 2001), 0-14% for BSE in cattle (Gore *et al.*, 1997), and 0% for vCJD in humans (Will et al., unpublished data). Other suggested routes include skin scarification (as demonstrated experimentally in mice (Taylor *et al.*, 1996)), food-borne infection via oral lesions (Bartz *et al.*, 2003), for scrapie possibly even mechanical transmission involving arthropods (Lupi, 2003), and for vCJD, iatrogenic transmission (Will, 2003). However, there is no evidence that exposure via any of these routes varies with age in a manner corresponding to the estimated risk of infection functions (Figure 4.2)

The measures of PP development (area, weight or number) used in this study are crude indicators of lymphoid tissue development; alternative measures in PP development may be at least as appropriate (for example, in sheep, counts of functionally mature FDCs). Moreover, this analysis assumes that both the anatomical data and the age-susceptibility estimates available are representative of each host species in general and not just the specific populations examined. Similarly, it is assumed that the associations studied have not been distorted by other factors (example, history of exposure to gut pathogens) which might influence PP development and/or susceptibility to TSEs.

Given these caveats, it is nonetheless striking that an association between PP development and susceptibility to TSEs is seen not just in one host species but in three host species with different relationships between these variables and age. This kind of comparative study is especially useful in cases such as this where experimental manipulations (example, of PP development) are not feasible.

Taken together, the epidemiological, anatomical and pathological evidence are consistent with the hypothesis that PP development or a close correlate of PP development is a major determinant of the observed age distribution of natural cases of TSEs in sheep, cattle and humans. This implies that the age groups most at risk of TSE infection (given that the individuals are exposed and have a susceptible PrP genotype) are indicated by the development of PPs in the gut.

Chapter 5: Ontogeny of PrP-associated follicular dendritic cells in postnatal mice and Cheviot sheep

5.1 Introduction

Prion protein (PrP), the product of the PrP gene, has been linked to TSE infection and disease progression based on the findings that the scrapie agent is unable to replicate in PrP knockout mice (Büeler *et al.*, 1993), and when PrP levels are increased by insertion of multiple PrP gene copies the disease progresses more rapidly (Prusiner, 1991). PrP^{Sc} deposits are associated with TSE pathogenesis and immunocytochemical detection of this pathological PrP isoform is routinely used as a hallmark of these diseases (Kitamoto *et al.*, 1987; Lantos *et al.*, 1992). After oral inoculation of rodents with scrapie, infectivity and PrP^{Sc} accumulate first in PPs and ganglia of the enteric nervous system before their detection in the central nervous system (CNS) (Beekes and McBride, 2000; Maignien *et al.*, 1999). In natural and experimental scrapie in sheep, PrP^{Sc} is often found to accumulate in PPs prior to detection within the CNS (Andréoletti *et al.*, 2000; van Keulen *et al.*, 1999). Within the gut-associated lymphoid tissue (GALT) of orally infected rodents (Beekes and McBride, 2000) and sheep (Andréoletti *et al.*, 2000; Heggebø *et al.*, 2000) with scrapie and mule deer inoculated with CWD (Sigurdson *et al.*, 2002), disease-specific PrP accumulations occur in association with FDCs. High levels of PrP have also been detected on FDCs in uninfected mice (Brown *et al.*, 2000a; Ritchie *et al.*, 1999). Although the precise identity of the gastrointestinal tract tissues and cells in which TSE infectivity is either supported or replicated remains to be established, the FDC appears to be the most likely candidate for prion replication.

FDCs are a unique population of accessory cells localized to primary and secondary nodules of lymphoid tissues (Heggebø *et al.*, 2002) and have been implicated as the likely sites for prion replication in lymphoid tissues as they appear to express high levels of abnormal PrP in patients with vCJD (Bruce *et al.*, 2001; Hill *et al.*, 1999), sheep with natural scrapie (van Keulen *et al.*, 1996) and mice experimentally infected with BSE (McBride *et al.*, 1992). Sub-lethal whole body γ-irradiation of mice before or after peripheral infection has no effect on scrapie incubation periods, suggesting involvement of non-proliferating, radioresistant cells in the lymphoreticular phase of prion propagation (Fraser and Farquhar, 1987). Amongst the best candidates for cells of this nature are FDCs. Signalling through TNF-R1 and LTβR is required for the development and maintainance of mature FDCs (Mabbott and Bruce, 2001). In spleens of TNFα-/- mice challenged with the ME7 scrapie strain, the absence of detectable infectivity and PrP^{Sc} in the spleen and reduced disease susceptibility coincide with the

absence of mature FDCs (Brown *et al.*, 1999; Mabbott *et al.*, 2000). Inhibition of LTβ signalling pathway with a soluble receptor that depletes FDCs (Mackay and Browning, 1998), prolongs the latency of scrapie after intraperitoneal (i/p) challenge (Mabbott *et al.*, 2000; Montrasio *et al.*, 2000), and B cell-deficient μMT mice (Kitamura *et al.*, 1991), which bear a deletion of the transmembrane μ-immunoglobulin exon, are resistant to i/p inoculation with prions (Klein *et al.*, 1998) perhaps because of impaired FDC maturation (Klein *et al.*, 1998; Montrasio *et al.*, 2000). SCID mice, which lack mature FDCs, are fully susceptible to TSE infection when challenged via the intracerebral (i/c) route, but are relatively resistant when exposed peripherally (Fraser *et al.*, 1996; Kitamoto *et al.*, 1991). Hence, although FDCs appear to play a major role in i/p infection, their contribution may not be essential in i/c route transmission (Kitamoto *et al.*, 1991).

In spite of oral exposure being a likely transmission route not only for vCJD but also for other TSEs, the majority of experiments that suggest involvement of PrP^{Sc} replication in FDCs have been performed using i/p routes of infection. Direct i/p inoculation is a potentially misleading model of natural TSE infection, and the role of FDCs following oral infection remains unsettled. For instance, the role of the spleen and of FDCs in amplification of prion infectivity following oral inoculation remains unclear, since, although splenectomy prolongs the incubation time of scrapie in intraperitoneally-infected animals (Fraser and Dickinson, 1970), it has no effect after oral or intragastric inoculation (Kimberlin and Walker, 1989a). Although the role of PrP^{C}-expressing mature splenic FDCs is well characterised at least in the FDC-dependent ME7/C57BL mouse scrapie model, the ontogeny of PrP^{C}-expressing FDCs in PPs and how this might affect susceptibility to TSE infection is not known. Previous studies have shown that neonatal mice are less susceptible to scrapie challenge compared to adults (Outram *et al.*, 1973). Although a proportion of neonates survived i/p infection, their ability to survive scrapie challenge disappeared in the first week of life and by 10 days post-partum, susceptibility to scrapie resembled that of the adult (Ierna, 2001). Because sheep are born with more developed lymphoreticular responses relative to mice, and possess an extensive bed of FDCs in the ileal PP at a time when they are considered to be most susceptible to infection (Heggebø *et al.*, 2000; Hourrigan *et al.*, 1979), this may help to explain the apparent increased susceptibility of young lambs and the decreased susceptibility observed in mice soon after birth. It is interesting to speculate that neonatal mice perhaps lack a mature cell population required for scrapie pathogenesis (Outram *et al.*, 1973) and when these cells undergo differentiation, susceptibility to scrapie is restored after a certain postnatal age. Because there is an intrinsic requirement for FDCs in

ME7 scrapie pathogenesis, this may suggest that maturation of FDCs may affect neonatal scrapie pathogenesis with this strain. Although previous studies suggest that FDCs are in an immature state during the first month of life because of immaturity of B cells (Marshall-Clarke *et al.*, 2000) and the inability of FDCs to trap immune complexes (Holmes *et al.*, 1984), other studies suggest that immature FDCs may be partially functional (Karrer *et al.*, 2000).

The majority of studies have focussed on the distribution of PrP in the spleen, lymph nodes and tonsil, easily accessible sites in comparison to the ileal PP. The aim of this chapter is, therefore, to investigate using immunocytochemical techniques the earliest age at which PrP^C can be detected in uninfected developing PP tissue in the ileum of C57BL mice and Cheviot sheep, and to verify its association with FDCs during ontogeny to determine any correlations with the onset of scrapie susceptibility in sheep and mice. Data obtained from this study may aid in explaining the apparent decreased susceptibility of neonatal mice and increased susceptibility in young sheep and whether this could be attributed to an absence of PrP-associated FDCs within PPs.

5.2 Materials and Methods

5.2.1 Mice

All mice used in this study were uninfected with scrapie and were bred in the NPU, Institute for Animal Health, Edinburgh. C57BL and $PrP^{-/-}$ mouse strains were used. The C57BL strain expresses normal levels of PrP^C and is used routinely at NPU for investigating the role of the lymphoreticular system (LRS) in scrapie peripheral pathogenesis and was used in experiments described in this chapter. $PrP^{-/-}$ mice, which do not produce PrP protein, were produced by insertion of an MT/Neo cassette into exon 3 of the PrP gene in 129/Ola mice (Manson *et al.*, 1994), and were used as a negative control in the ICC experiments.

5.2.1.1 Harvesting of mouse tissues

C57BL and $PrP^{-/-}$ mice of various age groups (2, 7, 14, 28 and 35 days) were culled using cervical dislocation. The small intestine was harvested and frozen sections prepared (see below). Each group comprised six mice for each of the two strains (Table 5.1). Because the intestine is a long organ, transverse sections are not sufficient and the organ is best studied longitudinally. A technique referred to as the 'Swiss roll', which has been described previously (Moolenbeek and Ruitenberg, 1981; Reilly and Kirsner, 1965), was used for examining the full length of the mouse ileum (Figure 5.1). The entire small intestine was

removed from the abdomen and dissected longitudinally. Faecal contents were removed and the intestine divided into three segments (the duodenum, jejunum and ileum) to facilitate easy handling. Each segment was rolled up longitudinally with the mucosa facing outwards. The 'Swiss roll' was snap frozen in liquid nitrogen (at -70°C) for cryostat sectioning. The 'Swiss roll' preparation necessitates rapid and careful removal of the intestine because within 10 minutes of killing the animal, there is considerable sloughing of the intestinal epithelium and loss of muscular tone.

Table 5.1 Mice used for investigation of PrP and FDC onset in ileal PP tissue.

	Mouse Strain	Age of mice				
		2 days	7 days	14 days	28 days	35 days
Number of animals	C57BL	6	6	6	6	6
	$PrP^{-/-}$	6	6	6	6	6

5.2.1.2 Preparation of frozen tissue

Snap frozen rolls of ileum were embedded in Tissue-Tek ®. Cryostat sections (7 μm) were cut at -20°C (see Appendix B-1 for full description of method) using electrostatically charged glass slides (Superfrost Plus glass®) that attracts frozen (and formalin fixed) tissue sections, binding them to the slide. The slides were dried overnight at room temperature, and then stored at -20°C in airtight boxes containing silica gel to help minimise humidity.

5.2.1.3 Double immunolabelling for PrP and FDCs

Cryostat sections were incubated with normal goat serum to block non-specific binding sites. Washing steps were performed with TBS. Sections were immunolabelled with a rabbit polyclonal anti PrP antibody, 1B3. 1B3, raised by inoculating rabbits with PrP^{Sc} fibrils from the brains of $Sinc^{s7}$ (PrP a/a) mice terminally affected with ME7 (Farquhar *et al.*, 1994; Farquhar *et al.*, 1989), recognises 4 distinct sites along the PrP peptide sequence including one site in the N-terminus region (Farquhar *et al.*, 1989; Korth *et al.*, 1997).

1B3 was applied at a 1:1000 dilution overnight at room temperature. To ensure antibody specificity, adjacent serial sections from the same tissue sample were incubated with normal rabbit serum in place of primary antibody. Immunolabelling was visualised using fluorescent goat anti-rabbit alexa 488 (1:200 dilution) (Invitrogen).

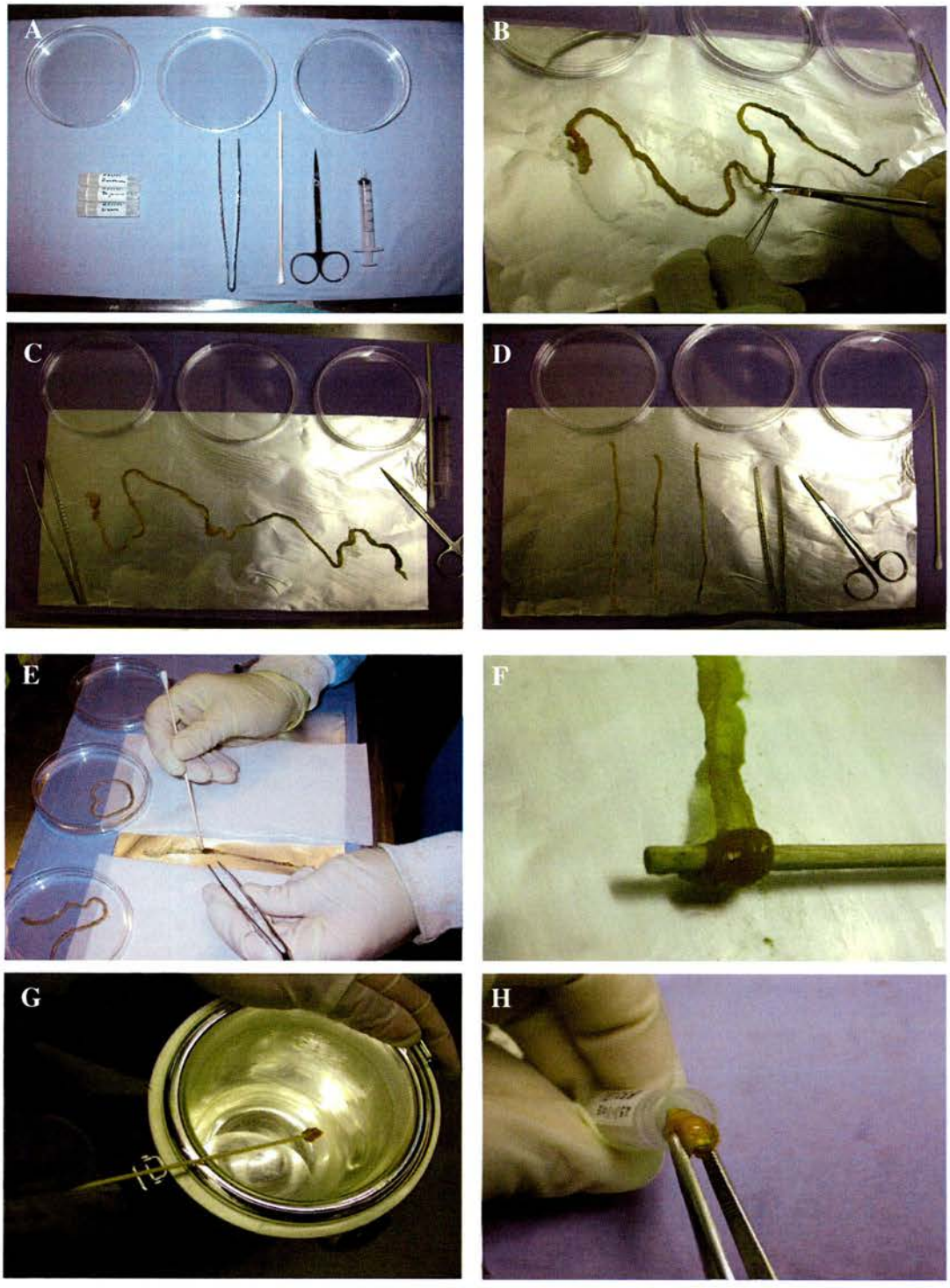

Figure 5.1 Preparing a 'Swiss roll' using the small intestine of the mouse. **A**. Materials for preparing 'Swiss roll'; **B-C**. Small intestine is slit open longitudinally with scissors from the caudal end; **D**. Divided intestine (duodenum, jejunum and ileum) with caudal ends nearest the investigator; **E**. Faeces are removed with a wooden stick. Segments are kept in normal saline to prevent desiccation; **F**. Cleaned segment is rolled onto stick with the mucosa facing outwards; **G**. 'Swiss roll' is snap frozen in liquid nitrogen (-70°C); **H**. 'Swiss roll' is carefully removed from stick and placed in a cryostat tube for ICC studies.

For FDC networks, sections were blocked with normal mouse serum and immunolabelled using a biotinylated monoclonal rat antibody, FDC-M2 (AMS Biotechnology Oxon, Abingdon, UK).

Recent studies have identified the antigen recognized by the FDC-specific antiserum FDC-M2 as complement component C4 (Taylor *et al.*, 2002). Normal rat serum was used for controls. Immunolabelling was visualised using streptavidin-alexa 594 diluted 1:200 in TBS (Invitrogen). Slides were mounted in fluorescent mounting media (DAKO) and examined under a confocal microscope. Immunolabelled slides were stored at 4°C to prevent photo-bleaching. The specificity of the double immunocytochemical labelling procedure was evaluated using healthy mouse spleen. (Full method described in Appendix B-2).

Double immunolabelled sections were analysed using confocal microscopy to determine areas of co-localisation between PrP^C and FDC markers. Co-localization analysis, based on intensity profiles, was used to further characterize double immunolabelling. Sections were viewed with green and red excitation/emission settings to detect PrP and FDCs, respectively. Immunolabelling intensity profiles were obtained by plotting the intensity (pixel intensity) of immunolabelling detected through the green and red channels versus the position of the labelling along a user defined line (XY position). The same process was repeated for $PrP^{-/-}$ mice. Superimposition of profiles for each channel was obtained for analysis. Merged green and red images appear yellow and indicate areas of co-localization.

5.2.2 Sheep

Thirty-three sheep (NPU Cheviots) aged 0-3, 3-6, 6-12, 12-18 and over 18 months were used in this study (see Appendix A, Table A-2). Their PrP genotypes were designated according to the major disease-linked polymorphisms at codons 136 (alanine/valine), 154 (arginine/histidine) and 171 (glutamine/arginine) (Hunter *et al.*, 2000). Genotype information was available for only 23 sheep. None of these sheep had any clinical evidence of disease. Sheep tissues were collected (as described in Chapter 3) for ICC and histoblot analysis from the ileum (that included several sites containing grossly identified PP tissue), and stored at -70°C in liquid nitrogen until use.

5.2.2.1 Single ICC using cryostat sections

Serial tissue sections were immunolabelled separately for PrP and FDCs. Cryostat sections (7 μm thick) were incubated with normal rabbit serum to block non-specific binding sites.

Staining for detection of PrP was carried out using the BG4 (primary) antibody (at a 1:100 dilution), a mouse monoclonal antibody (Jeffrey *et al.*, 2001a). Washing steps were performed with TBS. Streptavidin biotinylated alkaline phosphatase (1:100 dilution) was applied and colour development performed using Vector Red. To ensure antibody specificity, adjacent sections were incubated with normal mouse serum in place of primary antibody. (Full method is described in Appendix B-2). Images were taken using a Nikon E800 microscope linked to a Kodak Polaroid camera.

Immunolabelling for FDCs was carried out similarly on cryostat sections using the D62 (primary) antibody. (Full method is described in Appendix B-2). D62 was obtained from Bryan Charleston at Compton, Institute for Animal Health. The antibody has not yet been fully characterised.

5.2.2.2 Histoblots

Frozen sections of 10 μm in thickness were mounted on nitrocellulose membranes (0.45 micron pore size: Bio-Rad laboratories, Hemel Hempstead, Herts, England), and dried flat overnight at room temperature. After washing in TBS with 0.05% Tween, sections were digested with proteinase K (PK) solution (25 μg/ml) in PK buffer (10mM/L Tris HCl, pH 7.6; 100mM/L NaCl; 0.1% [w/v] Brij 35) for 3 hours at 37°C. After rinsing in distilled water, the samples were incubated in 3 mM Tris-buffered saline (with Tween) phenyl-sulphonylfluoride (TBST-PMSF) (at room temperature to stop PK activity), followed by denaturation in 3M guanidine isothiocyanate to denature PrP^C. 10% BSA was added to the membranes to block non-specific binding sites. Immunostaining was carried out using rabbit polyclonal antibody, 521.7 (van Keulen *et al.*, 1996) (at 1:30,000 dilution) to label PrP, and NBT/BCIP to visualise the reaction product. The control tissues were tonsil and mesenteric lymph node of a sheep with verified scrapie (positive control) and ileal PP tissue from an animal confirmed not to have scrapie (negative control). (Full method is described in Appendix B-2). Histoblots were analysed using a dissecting microscope.

5.3 Results

5.3.2 Mouse tissues

Using the 1B3/Alexa 488 system, PrP was found to be located within follicles of PPs and was first detected at 7 days in all six mice. No PrP^C was detected in PPs of 2-day old mice (not shown). Staining for PrP was also obtained in PP follicles of 14-, 28- and 35-day old mice and was more extensive compared to 7 day olds (with immunolabelling occurring most

extensively in 35-day old mice) (Figures 5.2 and 5.3). Not all PP follicles stained for PrP in some of these animals. PPs were difficult to locate in neonates (2-day olds).

Cells expressing FDC-M2 antigen, an epitope related to mouse C4 (Balogh *et al.*, 2001) were first observed at 7 days after birth, corresponding to the onset of detectable levels of PrP^C in developing PP follicles. FDC-M2+ cells appeared as well-defined networks and were localized to the follicular region. Although the morphology of FDC-M2 labelling at 7 days was very similar to that in the adult, staining appeared to be more diffuse in 35-day olds and covered a wider area of the follicle. None of the 2-day old mice demonstrated FDC-M2 reactivity in follicles (not shown). Although the immunolabelling technique was optimised to reduce background staining, FDC-M2 appeared to stain structures that resembled blood vessels (Figure 5.3O) in the lamina propria. No staining was observed in follicular regions sections incubated with normal rat serum suggesting that labelling was due to the presence of FDCs.

Confocal imaging of double stained sections showed some co-localisation between PrP and FDCs within follicles, surrounded largely by areas of single labelling for both PrP and FDCs. Yellow labelling was indicative of co-localization. In the case of 7-day old tissue for example, labelling detected through the green and red channels match each other closely between XY positions 150 and 210 μm (Figure 5.4A) but this corresponds to a minute area of co-localization as demonstrated by the yellow colour (Figure 5.2C). Overall, for each age group, the majority of PrP^C did not co-localise with FDC-M2, and there were many single labelled FDC-M2+ cells not expressing PrP^C. For example, the two red arrows in Figure 5.5G indicate two peaks of medium to high intensity in the red channel indicating PrP^C negative/FDC-M2+ cells. Co-localization appeared to be greatest for 35-day old mice (Figure 5.3O). There was little variation in the level of staining for each age group based on pixel density.

Age-matched controls ($PrP^{-/-}$) showed no staining for PrP in PPs (Figure 5.2(D and H) and Figure 5.3(L and P) as confirmed by line profile analysis (Figures 5.4 (B and D) and 5.5 (F and H). However, the overall intensity of FDC-M2 labelling in $PrP^{-/-}$ controls was lower compared to that observed for C57BL mice in all age groups (Figures 5.2 and 5.3), and this is again confirmed by line profile analysis (Figures 5.4 and 5.5).

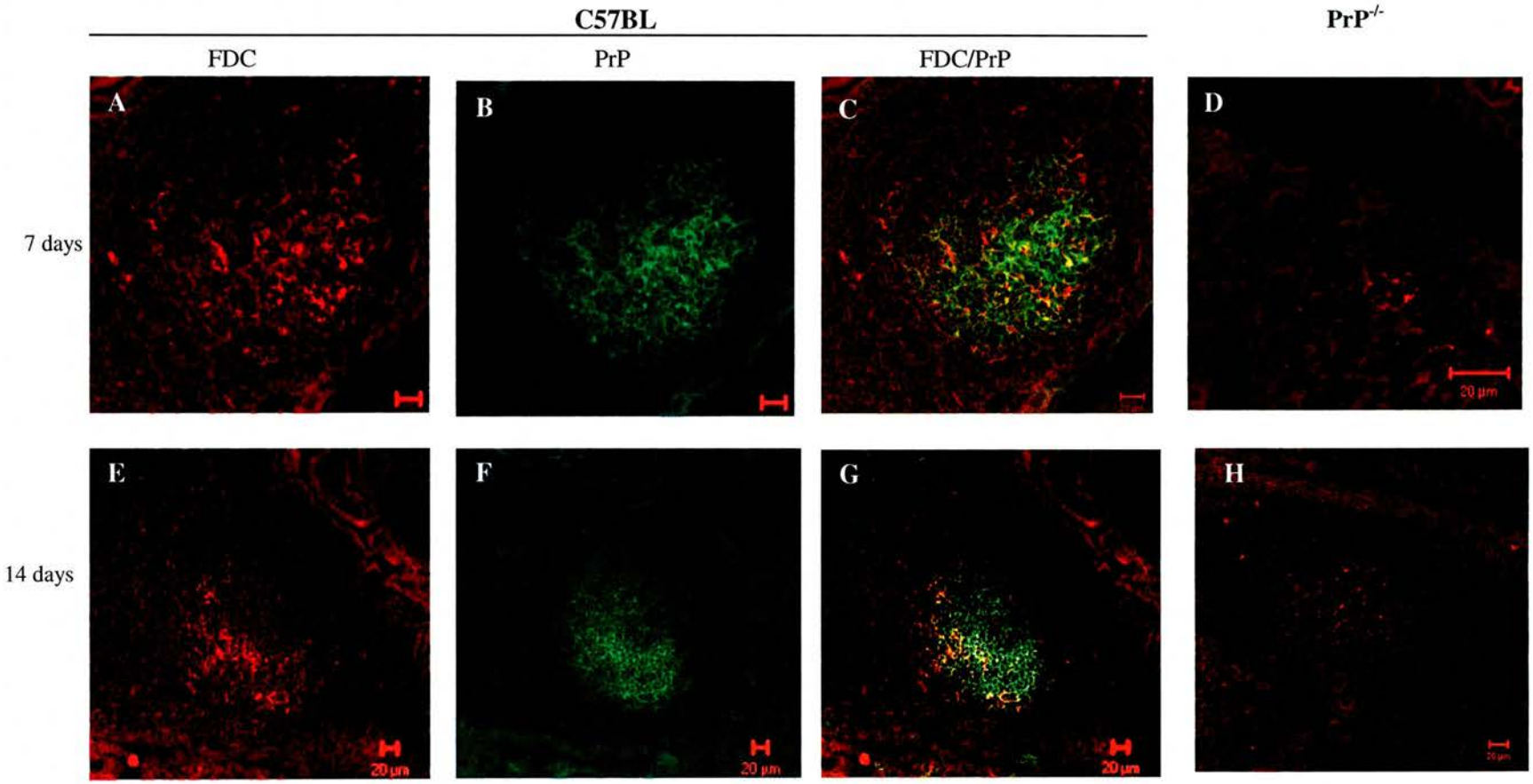

Figure 5.2 (A-H): Confocal imaging of ileal PP sections in 7 and 14 day-old C57BL and $PrP^{-/-}$ mice. Frozen sections were immunolabelled for FDCs and PrP using the antibodies FDC-M2 and 1B3, respectively (as described in section 5.2.1.3). FDC labelling was detected using streptavidin-alexa 594 (red) and PrP labelling was detected using goat anti-rabbit conjugated Alexa 488™ (green). Co-localization of FDC-M2+/PrP+ cells are indicated by yellow areas where red and green labelling overlap.

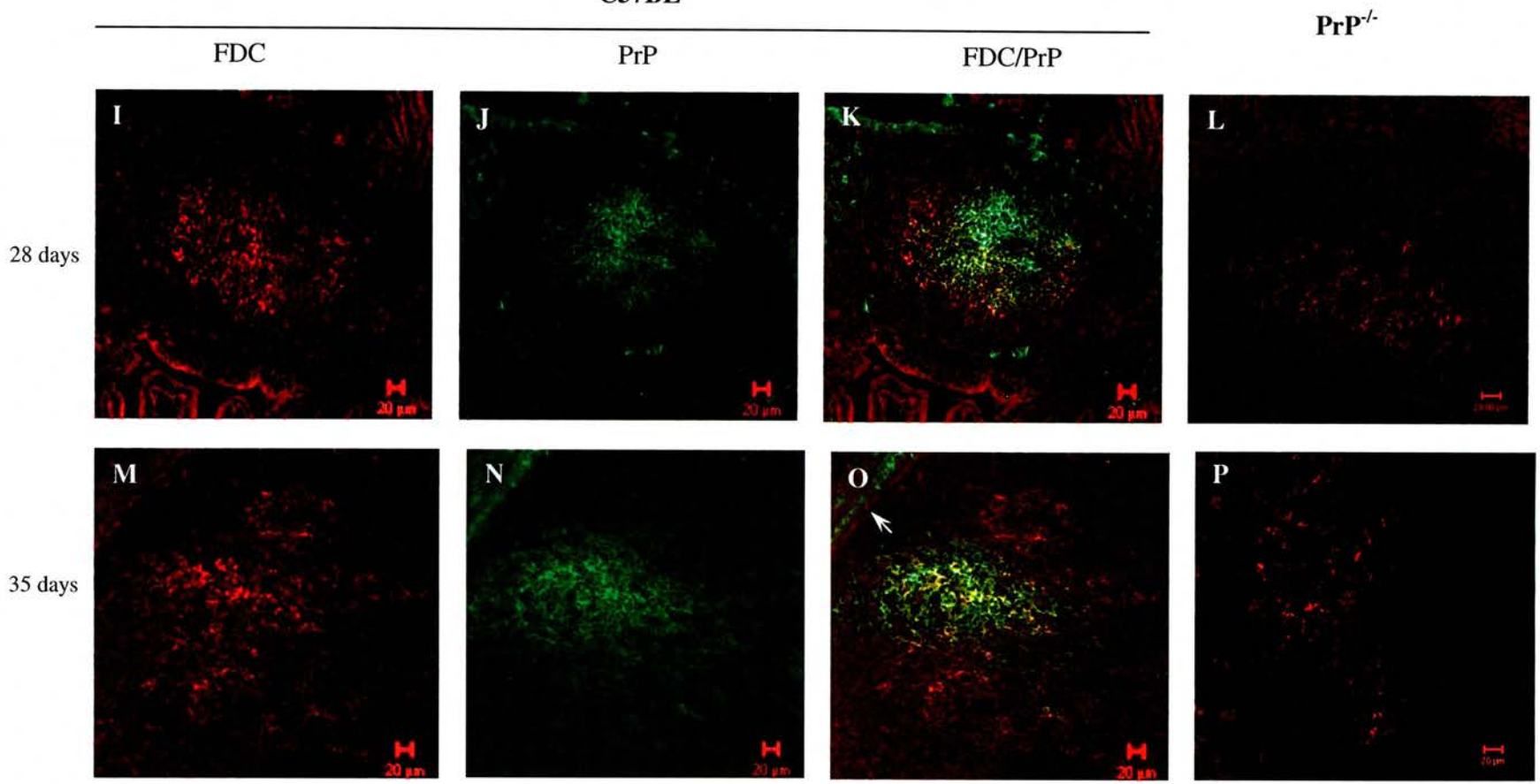

Figure 5.3 (I-P): Confocal imaging of ileal PP sections in 28 and 35 day-old C57BL and $PrP^{-/-}$ mice. Frozen sections were immunolabelled for FDCs and PrP using the antibodies FDC-M2 and 1B3, respectively (as described in section 5.2.1.3). FDC labelling was detected using streptavidin-alexa 594 (red) and PrP labelling was detected using goat anti-rabbit conjugated Alexa 488™ (green). Co-localization of FDC-M2+/PrP+ cells are indicated by yellow areas where red and green labelling overlap. Arrow in **Figure 5.3 O** indicates labelling in a structure resembling a blood vessel.

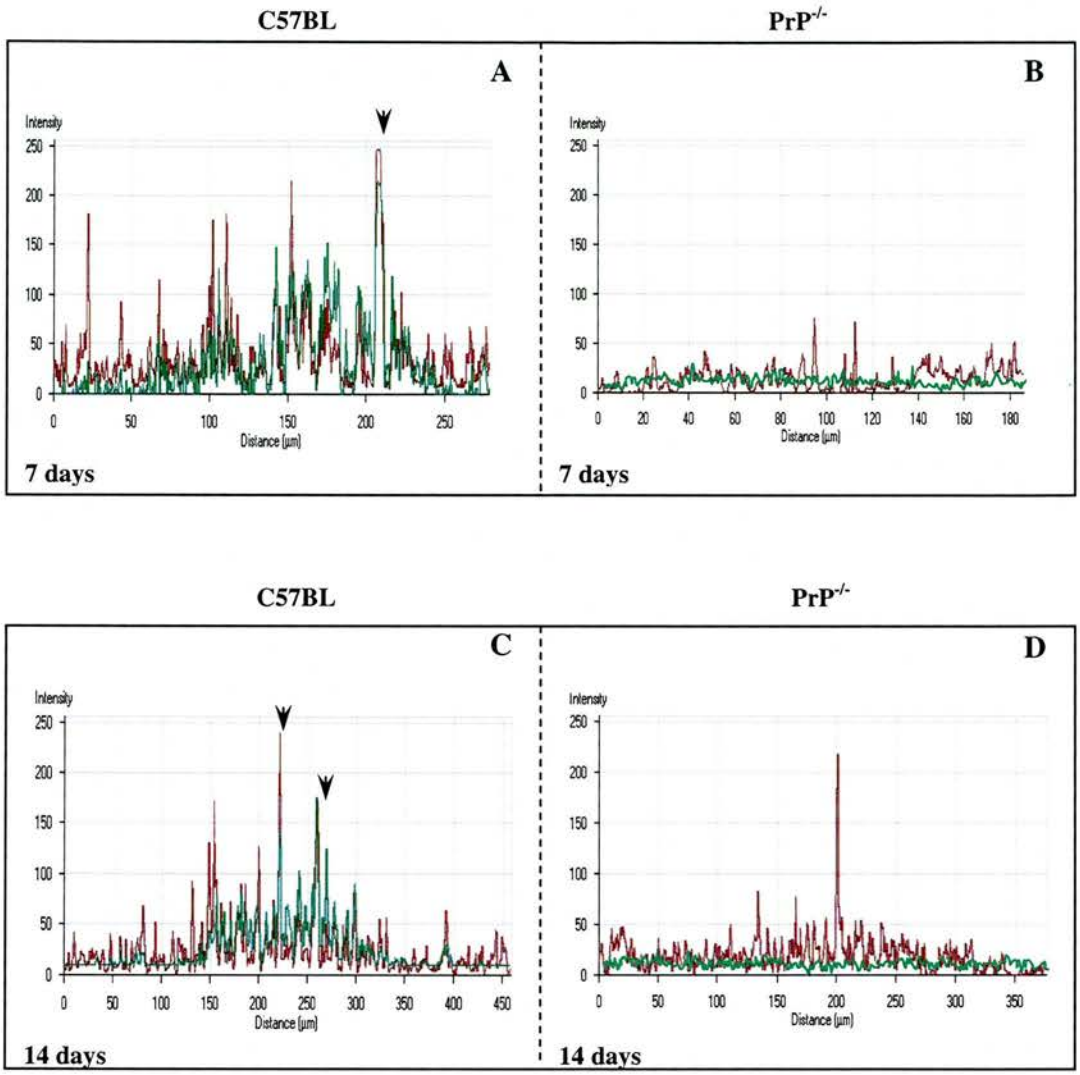

Figure 5.4 Co-localization analysis of 7 (**A** and **B**) and 14-day old (**C** and **D**) ileal PPs in mice, double-immunolabelled for FDCs (red) and PrP (green). Pixel intensities for FDC and PrP labelling correspond to FDC/PrP co-localization in Figures 5.2 for C57BL (**C** and **G**) and $PrP^{-/-}$ (**D** and **H**). Immunolabelling intensity profiles were obtained as described previously (section 5.2.1.3). Areas of co-localization are indicated by close matching of XY positions for red and green labelling. Red arrows indicate areas of single labelled FDC-M2+ cells. Black arrow heads indicate areas of co-localization.

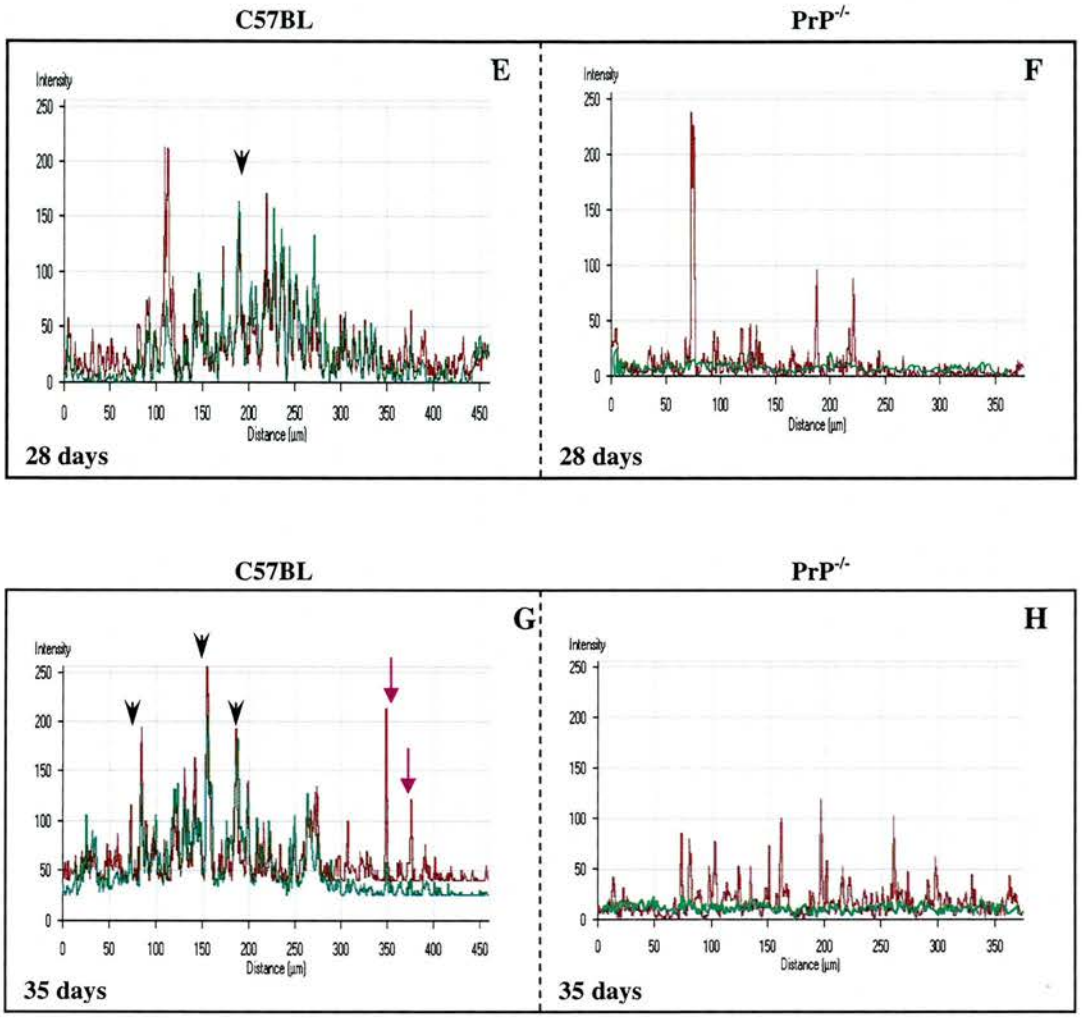

Figure 5.5 Co-localization analysis of 28 (**E** and **F**) and 35-day old (**G** and **H**) ileal PPs in mice, double-immunolabelled for FDCs (red) and PrP (green). Pixel intensities for FDC and PrP labelling correspond to FDC/PrP co-localization in Figures 5.3 for C57BL (**K** and **O**) and $PrP^{-/-}$ (**L** and **P**). Immunolabelling intensity profiles were obtained as described previously (section 5.2.1.3). Areas of co-localization are indicated by close matching of XY positions for red and green labelling. Red arrows indicate areas of single labelled FDC-M2+ cells. Black arrow heads indicate areas of co-localization.

5.3.3 Sheep Tissues

The PrP genotypes of sheep used in this part of the study are given in Appendix A, Table A-2. PrP genotypes are designated according to the major disease-linked polymorphisms at codons 136 (alanine/valine), 154 (arginine/histidine) and 171 (glutamine/arginine). Whether or not patterns of staining were similar for different genotypes within each age group could not be determined because of small sample size.

5.3.3.1 ICC

5.3.3.1.1 Staining for PrP and FDCs using cryostat sections

Consecutive ileal sections were labelled with the BG4 and D62 antibodies because simultaneous staining had not succeeded. Staining for PrP was detected in all age groups investigated. The level of staining, however, differed between follicles and between individuals. There were accumulations of PrP in most nodules of PPs in the ileum and the distribution of staining was similar in all age groups of sheep (Figure 5.6). Staining was observed primarily in the light central zone of follicles with little/faint or no staining present in the dark peripheral zone adjacent to the capsule.

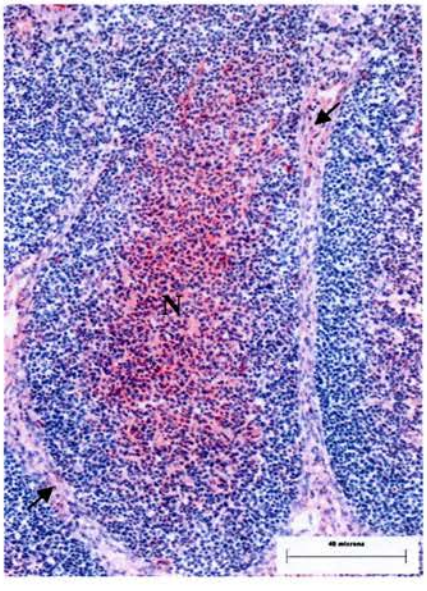

Figure 5.6 Distribution of PrP in a cryostat section of ileal PP tissue of an uninfected 9 month-old sheep using the BG4 antibody. Staining is confined mainly to the central region of the lymphoid follicle in the region of the FDC networks (N). Some faint background staining is also visible in the interfollicular regions (arrows).

There was a diffuse pattern of staining observed (using the D62 antibody) within the light zone of follicles, corresponding to the localization of FDCs and characterized by a reticular-like pattern (Jeffrey *et al.*, 2000b). Staining was detected in all age groups investigated (Figure 5.7). However, staining in old sheep (over 18 months) was found to be less diffuse and less intense (Figure 5.8). Dense staining was sometimes observed in the luminal border of the epithelial cells in the FAE (Figure 5.8). Dense, large, punctuate-type staining was sometimes found to be present in the dome and interfollicular regions of some follicles (Figure 5.7). However, similar staining was also observed in control sections using normal serum, and was most likely the result of artefact/non-specific staining.

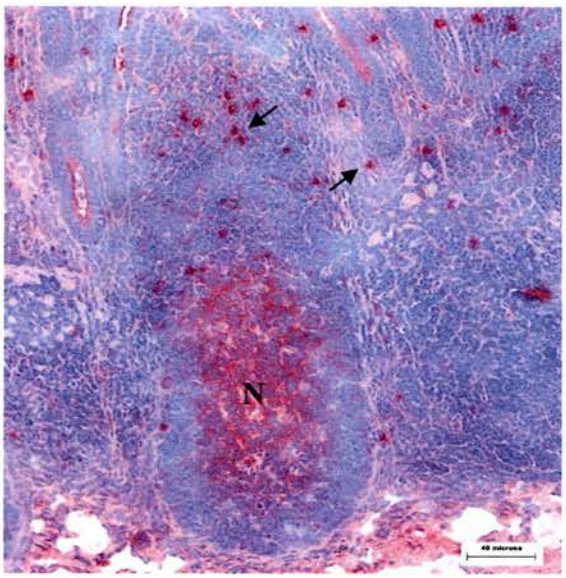

Figure 5.7 Distribution of FDCs in a cryostat section of ileal PP tissue of an uninfected 9 month-old sheep using the D62 antibody. Note the reticular-like network of these cells in the central region of the follicle (N). Dense punctuate areas of labelling represent non-specific staining (arrows).

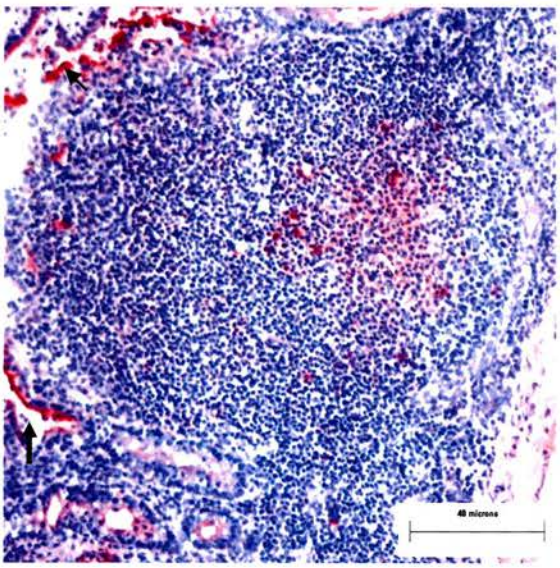

Figure 5.8 Distribution of FDCs in a cryostat section of ileal PP tissue of an uninfected 6 year-old sheep using the D62 antibody. The FDC network appears to have degenerated with age (N). Areas of dense staining are visible in the luminal border of some epithelial cells (arrow).

The antibodies BG4 and D62 tended to produce relatively high background staining in frozen tissue sections despite the fact that the immunolabelling technique was repeated and optimised to minimise cross-reactivity. Staining, though faint, was also observed in similar regions of control sections incubated with normal serum and suggests that labelling was not entirely specific for PrP and FDCs.

5.3.3.1.2 Histoblots

Immunocytochemical techniques, using cryostat sections, did not differentiate between the cellular (PrP^{C}) and disease-associated isoform (PrP^{Sc}) of the protein. The histoblot technique is useful for differentiating between PrP^{Sc} andPrP^{C}. The technique has the advantage in that it preserves the tissue anatomy to some degree thereby revealing the distribution of PrP^{Sc} within tissues {Heggebø *et al.*, 2003) as observed in this study. Staining was more intense for PK-positive samples compared to PK-negative samples (Figure 5.9). Based on histoblots, only one sheep of PrP genotype VRQ/VRQ was found to be subclinically infected with scrapie. Uninfected sheep had the genotypes ARR/ARR, ARR/AHQ, VRQ/AHQ or AHQ/AHQ. In this study, PrP^{Sc} was detected in ileal PPs in one 9-month old sheep (Figure 5.9D). Proteinase K-resistant PrP was detected in the lymphoid nodules and was localized mainly to the light central zone and neck region of the nodules. The dark peripheral zone showed little staining and there were scattered foci of strong staining in nodules perhaps corresponding to tingible body macrophages (Appendix C, Figure C-1). This was in contrast to staining observed in uninfected animals which appeared to be less intense and which showed no specific punctuate-type pattern in lymphoid nodules or extranodular regions (Figure 5.9B). Histoblot and immunocytochemical examination of negative control animals showed no PrP^{Sc} in the ileal PP tissue (Figure 5.9F). There was dark, granular staining in the germinal centres in the mesenteric lymph node of positive controls (Figure 5.9H).

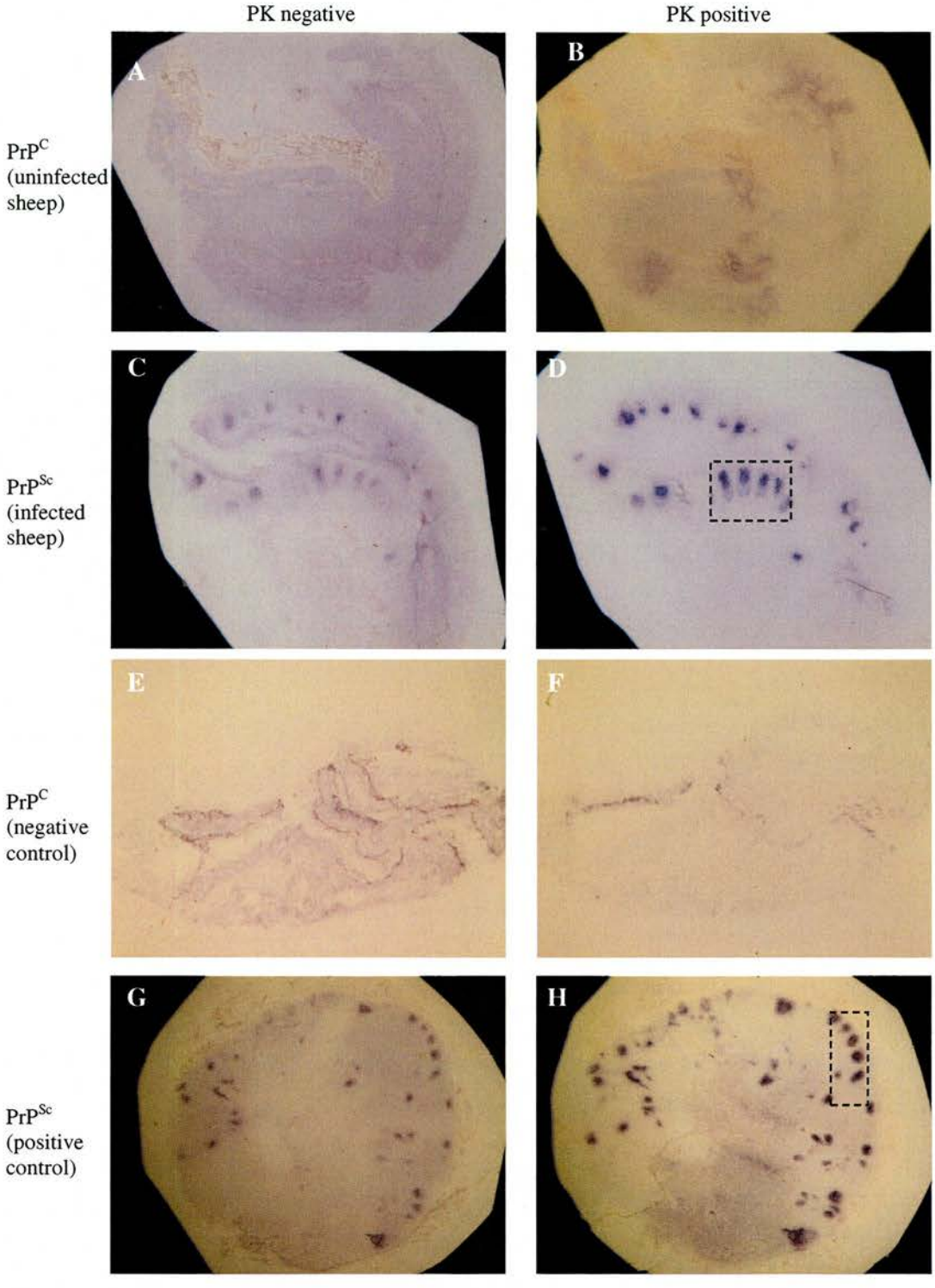

Figure 5.9 Histoblots of ileal PP tissue and mesenteric lymph node. Magnification X10. Histoblots from an uninfected animal in TBS (PK negative) **(A)** and PK solution (PK positive) **(B)**. Histoblot analysis of PrP accumulation in ileal PP tissue from a VRQ/VRQ sheep (9 months old) with natural scrapie infection **(C** and **D)**. Histoblot of the ileal PP in an uninfected animal, used as a negative control in this study **(E** and **F)**. Histoblot of the mesenteric lymph node from a scrapie-infected animal, used as a positive control in this study **(G** and **H)**. (See also Appendix C, Figure C-1 for explanation of 'boxed' regions in **Figure 5.9D** and **H)**.

5.4 Discussion

5.4.1 Mice

Double immunofluorescence was used to investigate the co-localisation of PrP and FDCs in frozen sections of ileal tissue in uninfected mice. This study does not assume that cells associated with the cellular expression of PrP (PrP^{C}) necessarily participate in TSE replication (although it would seem likely that PrP^{Sc} accumulations in specific cell types may indicate their involvement in TSE pathogenesis).

In this study, it was possible to identify FDCs in PPs of the mouse ileum using a specific monoclonal antibody (FDC-M2). PrP labelling was present in all age groups of mice, except the 2-day old category, in the centre of lymphoid follicles. Other authors have used the FDC-M2 antibody in determining the association between PrP and FDCs in splenic mouse tissue (Brown *et al.*, 1999; Kosco *et al.*, 1992). The overall intensity of FDC-M2 labelling in $PrP^{-/-}$ PPs was found to be lower than that of C57BL PP tissue and is in accordance with findings from a previous study using splenic tissue from mice (Ierna, 2001). This finding suggests that FDCs may undergo a different rate of maturation in $PrP^{-/-}$and C57BL mice perhaps because of their genetic backgrounds. Further investigation will be required in order to determine whether or not this is a significant finding.

Previous studies have shown that by day 10, few splenic follicles contain cells expressing the FDC-M2 antigen. However, the earliest FDC marker displayed was the antigenic determinant recognized by the FDC-M1 monoclonal antibody, which was detectable by day 3 prior to follicle formation in splenic cells (Balogh *et al.*, 2001). The FDC-M1 marker was not used in this study because it also stains tingible body macrophages which are most likely of haemopoietic origin (Aguzzi *et al.*, 2003). Although FDC-M1 has been cited as a maturational marker for FDCs (van den Berg and Dijkistra, 1995), it has not been sequenced and its function has not yet been determined.

In this study, FDCs were detected significantly earlier than in other lymphoid structures using the FDC-M2 marker (Ierna, 2001). The appearance of FDC-M2+ cells at 7 days old may suggest that FDCs are functionally mature at this age or are reaching functional maturity. It is interesting that the onset of FDC-M2 labelling follows the onset of detectable levels of PrP, an observation perhaps suggestive of the intricate involvement of PrP in FDC maturation. However, there was little co-localisation between PrP and FDCs in ileal PP tissue suggesting that, in addition to FDCs, other cells like macrophages or interdigitating

dendritic cells (Andréoletti *et al.*, 2000) may also be involved in oral scrapie pathogenesis in mice. Little co-localization between PrP and FDCs may also suggest that PrP-expressing FDCs are at different maturational levels, with single labelled FDCs not having acquired full maturational status. Findings in this study are in contrast to the findings of a previous study where it was found that in adult (30-day old) mice, most of the PrP^C labelling within splenic follicles co-localized with FDC-M2+ cells and no single FDC-M2+ cells were observed. A significant degree of co-localization between PrP and FDC-M2+ cells was also found in splenic follicles of 7-, 10- and 14-day old mice (Ierna, 2001). Similar findings (in splenic tissue) were reported using the FDC-M1 antibody (Brown *et al.*, 1999; Ritchie *et al.*, 1999). Differences between observations made in this present study and previous studies suggest that splenic FDCs may play a potentially more important role in TSE pathogenesis than FDCs that reside in follicles of PPs based on association of these cells with PrP. Alternatively, infectivity may reside in other cell types (like macrophages) until FDCs have reached a certain maturation state. The possibility that other cells present in PPs may play a more intricate role in TSE pathogenesis exists and may render FDCs less critical (or not absolutely essential) for propagation of the agent.

In this study, the distribution of FDC-M2 indicates that, in addition to FDCs, uncharacterized reticular and perivascular cells may also be involved in the capture and possibly also the retention of immune complexes and antigens (Taylor *et al.*, 2002). This question may be addressed by using other markers to investigate the co-localization of PrP and leukocyte populations, including macrophage and dendritic cell lineages in extra-nodular tissue. Results from previous studies exclude the idea that FDCs (or B cells) are absolutely necessary for infection (including oral infection) and spread to the brain, and suggest that other cell types, including isolated lymphoid follicles (ILFs) (see Chapters 1 and 6) macrophages or dendritic cells, may play a more important role in TSE pathogenesis than is generally appreciated (Shlomchik *et al.*, 2001). In this present study, cells located at the periphery of lymphoid follicles could not be considered as FDCs since they did not exhibit the typical dendritic shape nor were they located within germinal centres (Imai *et al.*, 1998). Similar observations have been made using the FDC-M1 antibody, used to identify murine FDCs, and that cross-reacts with some macrophages (Montrasio *et al.*, 2000) as mentioned previously. FDCs have also been identified functionally by their ability to trap intravenously injected immune complexes (van Rooijen and Streefkerk, 1976) and it has been found that the ability of FDC-M1 positive cells to trap immune complexes coincides with the

appearance of CD21/35 (complement receptor Type 2 and 1, CR1.2) receptors on cells with a follicular location in spleen tissue (Balogh *et al.*, 2001).

At 35 days, labelling was not confined to the central region of the germinal follicle but was relatively diffuse and covered a wide area of the follicle. The diffuse nature of this staining may suggest morphological differences between the dendritic processes of FDCs of young mice (i.e. 7 days) and older mice (35 days). Ultrastructural studies have shown that mature mouse spleen FDCs residing within the light zone of the follicles appear to have long cytoplasmic extensions, and could be distinguished from FDCs residing within the dark zone, which have less pronounced extensions (Jeffrey *et al.*, 2000a). Imai *et al.* (1986) have shown that the transition between immature and mature FDCs with labyrinthine structures occurred between 28 and 35 days after birth. It is possible, however, that the route, type and amount of environmental antigenic stimulation and the type of lymphoid tissue studied may affect the development of germinal centres.

It has been reported that young mice become fully susceptible to peripheral scrapie infection around the time at which FDCs in splenic tissue are detected and that FDC-M2 may detect FDCs at a critical stage in maturation required for TSE pathogenesis (Ierna, 2001). Future studies need to be performed to determine if young mice also become fully susceptible to oral TSE infection around the time in which FDC-M2 labelling is observed in the PPs of the ileum. If so, it may be speculated that immaturity of FDC networks in PPs may hamper or delay scrapie replication or prevent it completely, following oral inoculation of the agent. It has been suggested that quantitative differences may also exist between the amount of PrP^C in young and adult spleens (Ierna, 2001), based on the area of tissue that was immunolabelled for the protein. A similar observation was also made for PP tissue in this present study and may be partly responsible for the greater susceptibility to peripheral scrapie infection observed in adult mice. Mice used in this study were not infected and whether or not the distribution of PrP labelling is the same in PP tissue from scrapie-infected and uninfected mice is not known. Previous immunocytochemical studies have shown, however, that host PrP is localised to FDCs in the spleen of normal and scrapie infected mice (McBride *et al.*, 1992; Ritchie *et al.*, 1999) and strong, granular staining has been reported within germinal centres (where FDCs reside) in spleen tissue of infected mice (Ritchie *et al.*, 1999). Studies in mice have also shown that PrP-expressing FDCs are needed for disease without any direct involvement of bone-marrow derived cells such as lymphoid or myeloid

cells (Brown *et al.*, 1999). However, these studies were performed using peripheral routes of infection and only spleen tissue was examined in young adult mice.

It is important to bear in mind that cellular mechanisms of TSE pathogenesis and the importance of lymphoreticular amplification could differ depending upon the species, dose and strain of infecting agent and the route of inoculation used. It has been found, for instance, using a murine scrapie model, that splenectomy had no effect after oral challenge, although infectivity was found in PPs (Kimberlin and Walker, 1989a). Hence, although the spleen appears to be a major site of TSE replication and an important route of entry into the CNS following peripheral infection (Kimberlin and Walker, 1989a), its role in oral TSE pathogenesis may not be as well established.

5.4.3 Sheep

This study yields further insight into the distribution of PrP in PP tissue of the ileum of sheep. PrP^{C} was found to be associated with the light zone of lymphoid nodules. Conventional immunocytochemical approaches are a useful tool in this type of investigation but wider application of these approaches to methods like histoblotting and PET-blotting (Schulz-Schaeffer *et al.*, 2000a; Schulz-Schaeffer *et al.*, 2000b) are necessary to distinguish between the normal cellular form of PrP (PrP^{C}) and the abnormal isoform associated with scrapie (PrP^{Sc}). Conformational changes associated with PrP^{Sc} confer a relative resistance to proteinase digestion, which is used as a basis for distinguishing between PrP^{C} and PrP^{Sc}. Furthermore, histoblots are useful since they retain some degree of preservation of tissue anatomy and are therefore able to demonstrate the distribution of PrP^{Sc} in tissues.

Interestingly, staining in histoblots appeared to be more intense in PK positive samples compared to PK negative samples. However, the significance of this staining is at present uncertain. It may be attributed to more epitope sites on the peptide chain being exposed for binding by primary antibody following protein kinase treatment, it may represent a non-specific binding feature of antibodies to macrophages (which may be enhanced during infection) or it may signify a specific up-regulation of PrP^{C}-bearing cells in disease (Heggebø *et al.*, 2002). Histoblots showed that PrP^{Sc} (proteinase K-resistant PrP) was confined mainly to the follicles/lymphoid nodules and more precisely in the light zone where FDCs are localized. Staining appeared as a granular punctate pattern confined to follicular regions. In uninfected animals, no punctuate-type staining was found to be associated with follicles. The reason for this variation in staining is not known, although it suggests, most

likely, involvement of the scrapie agent and its association with the follicular region. Interpretations, however, *cannot* be made based solely on observations from one infected sheep. Whether the variation in staining observed between individuals was also the result of differences in PrP genotype is unknown.

In sheep, specific antibodies recognising FDCs are lacking. D62, which has been previously shown to be able to identify FDC populations in other species (including humans) using secondary lymphoid tissue (Bryan Charleston, unpublished), was used to identify FDCs in sheep ileum. However, the antibody has not as yet been fully characterised. In this study, labelling for FDCs (which occurred as fine, diffuse, reticular staining) and PrP occurred in the majority of follicles of the distal ileum of all age groups of uninfected sheep investigated and was confined largely to the centre of lymphoid nodules. Labelling varied in intensity within and between individual follicles and between individual sheep. A relatively high level of background staining was observed in frozen sections stained with BG4 or D62. Since cryostats preserve the antigenicity of PrP, background staining may be attributed to cross-reactivity between BG4 (and D62) and non-PrP (and non-FDC) epitopes present on other cells or tissue structures such as blood vessels. Although an association between PrP accumulation and FDC network in lymphoid follicles in PPs was not specifically demonstrated through double immunolabelling, this association can only be presumed on the basis of similarities between PrP immunolabelling and morphology and localization of FDCs in lymphoid follicles (Andréoletti *et al.*, 2000; van Keulen *et al.*, 1996). Findings in this present study suggest that FDCs may be a significant site of accumulation of PrP in sheep and is in agreement with results from previous studies (Heggebø *et al.*, 2002). Furthermore, the presence of mature FDC networks in the gut of newborn lambs raises the question of maternal transmission of the infectious agent from ewe to lamb as a result of exposure to infected placental tissue.

It has been shown that lymphocyte function decreases with age, and this will result in degeneration of FDC networks (Kosco-Vilbois, 2000). In older sheep (over 18 months of age) all follicles had undergone involution, and although visible, appeared to be hypocellular and shrunken as described in Chapter 3 in this thesis and by other authors (Reynolds and Morris, 1983). Whether or not degeneration of FDC networks (Figure 5.8) can be correlated to their ability to accumulate and replicate prions is not known. However, if the number of functional FDCs is significantly reduced in older animals, this may well contribute to reduced susceptibility to TSE infection observed in older animals.

Co-localization and immunocytochemical staining in tissues is informative, but they are not the most sensitive or quantitative methods of protein detection. Furthermore, whether PrP^C location and distribution are wholly or partially indicative of the extensiveness of this PrP during disease is not known. To determine whether FDCs and (other cells) are plausible candidates for infectious PrP replication, *in vitro* experiments that maintain these cells in culture are required (Sy and Gambetti, 1999). Furthermore, because PrP^C is expressed at high levels in human leukocytes (including T cells, B cells, monocytes and dendritic cells) compared to murine leukocytes (Liu *et al.*, 2001), it is possible that these cells may well have different roles in TSE pathogenesis in the two species. Whether or not observations made in murine models are directly relevant to human prion disease can only be determined through additional studies. Until then, the identity of cell types responsible for prion replication will remain controversial.

The emergence of vCJD in humans, most likely due to consumption of BSE-contaminated meat and meat products (Bruce *et al.*, 1997), and the young age distribution of vCJD cases has provided renewed impetus to understanding the pathogenesis of oral transmission of the naturally occurring TSEs (including scrapie, BSE and CWD) and the apparent age-dependent susceptibility to these diseases. For natural scrapie, incidence in sheep flocks tends to peak in young adult years despite different forces-of-infection and considerable differences in disease incidence (Detweiler and Baylis, 2003; Redman *et al.*, 2002). A similar age distribution has been observed for BSE in cattle with the majority of cases occurring in animals aged 5 to 7 years (Anderson *et al.*, 1996), and data on prevalence of CWD suggest that an age-specific peak exists for CWD in free-ranging cervids in Colorado and Wyomming (Heisey and Joly, 2004; Miller *et al.*, 2000).

That young people were more exposed to BSE-contaminated beef and beef products is not sufficient to explain the strikingly high proportion of vCJD cases among adolescents and young adults, and to account for the current age distribution of vCJD cases, an additional effect of age-dependent susceptibility is required (Boëlle *et al.*, 2004). Although natural TSE infection occurs most likely via the oral route (Detweiler and Baylis, 2003; Heggebø *et al.*, 2000; Sigurdson *et al.*, 1999; Terry *et al.*, 2003; Will, 2003), the intraperitoneal (i/p) and subcutaneous (s/c) routes of infection have been used in the majority of susceptibility studies and may provide potentially misleading models of natural oral infection. In intracerebral (i/c) challenge experiments, TSE infection may bypass the immune system portal, including Peyer's patches (PPs), which are known to play a key role in the infection dynamics of a range of TSEs, making it difficult to assess disease risk if the gut immune system is involved in oral TSE pathogenesis.

The main aim of this thesis was, therefore, to investigate the potential role of PP tissue in determining age-dependent susceptibility to natural TSE infection. Here, results from the preceding chapters are discussed in the context of age-dependent susceptibility to the TSEs and implications for future research are considered.

Because oral inoculation has been recognised as a relatively inefficient mechanism of TSE transmission, this may help to explain why a relatively small number of individuals have so far developed clinical signs of vCJD despite the possibility that a large proportion of the UK

population may have been exposed to BSE-contaminated material. However, complexities arise because infection via the oral route may not necessarily result in clinical signs of terminal disease, and knowledge of host factors does not always make it possible to predict with certainty which individuals will develop disease. For example, not all sheep from the positive selection line and comprising susceptible genotypes developed disease following oral TSE exposure in this study (Chapter 2). This observation parallels findings in other studies where only a proportion of genetically susceptible sheep were found to develop scrapie following oral dosing or when residing in naturally infected flocks (Foster *et al.*, 2001; Jeffrey *et al.*, 2001b; O'Rourke *et al.*, 1997). Findings imply that, in addition to genetic factors (Hunter *et al.*, 1996), other factors including agent characteristics (Goldmann *et al.*, 1994), as well as environmental factors (Elsen *et al.*, 1999; Healy *et al.*, 2004), route of exposure, level of infectious challenge (Jeffrey *et al.*, 2001b) and age at infection (Diaz *et al.*, 2005) may well be involved in the control of natural and experimental disease.

Age at exposure to the TSE agent was found to be a potentially important factor in determining whether an animal developed scrapie and the incubation period of the disease. Because younger animals appear to be more susceptible to scrapie and seem unlikely to develop the disease if they have not already done so by one year post-infection (Chapter 2), this may explain the age distribution of scrapie cases. One interesting finding in this present study was that younger sheep infected subcutaneously with a high or low dose of the TSE agent generally had shorter incubation periods compared to older animals (over 18 months) infected via the same route (Chapter 2).

Experiments carried out by Outram in the 1970s (Outram *et al.*, 1973) have shown that following s/c challenge, a high proportion of neonatally infected mice developed scrapie after incubation periods that were significantly shorter than that of adults. Although the involvement of the LRS could not be excluded since experiments were performed using immunocompetent mice, similar results were obtained more recently using ME7 infection of SCID mice in which the LRS component is impaired. In addition, the number of cases with short incubation periods decreased significantly following s/c inoculation of mice of 14 days or older (Ierna, 2001). These findings imply that immature nerves may be vulnerable to scrapie infection. Peripheral innervation is greater in neonates compared to 14 day old mice (Brown *et al.*, 1976; Lichtman, 1977) and PrP^C levels are also high in developing axons and during synaptogenesis (Sales *et al.*, 2002), either of which may facilitate uptake and transmission of infectivity. Interestingly, peripheral nerves remain under-developed until two weeks after birth (Jessen and Mirsky, 1999) during which time myelinating Schwann cells,

which encircle and protect the axon from damage and infection (Mirsky and Jessen, 1999) are immature and not yet fully functional. This is consistent with previous findings, which have shown that incapacitated nerves result in shortened scrapie incubation periods (Kimberlin *et al.*, 1983a; Kimberlin *et al.*, 1983b). In support of this observation, Glatzel *et al.* (2001) showed that mice with hyperinnervated lymphoid organs developed scrapie more readily following peripheral challenge with the RML strain. The enteric nervous system is suggested as the site of initial neuroinvasion for the scrapie agent (Heggebø *et al.*, 2000; van Keulen *et al.*, 1999; Van Keulen *et al.*, 2000). Evidence implies that the ileal PP may be a significant portal of entry for the TSE agent from the alimentary tract and that accumulation of the agent in these lymphoid organs may facilitate neuroinvasion (Lasmézas *et al.*, 1996). Although studies have suggested that lymphoid nodules are poorly innervated structures (Balemba *et al.*, 1998), a recent investigation of the distribution of the enteric nervous system in PP of scrapie-affected Suffolk sheep showed an extensive network of nerve fibres within many lymphoid nodules (Heggebø *et al.*, 2003) that were found to be in close association with FDCs and tingible body macrophages harbouring abundant PrP^{Sc}. This close association suggests that PP nodules are the sites of neuroinvasion and may preclude the need for non-neuronal PrP^{Sc} transport away form germinal centres (Press *et al.*, 2004).

Specific strains of the TSE agent may preferentially affect specific cell types. In Chapter 2, BSE was used in the majority of oral inoculations. Interestingly, young and old animals displayed similar incubation periods when inoculated with this agent via the oral route. In contrast, SSBP/1 was primarily used for s/c exposure, and in this instance, young animals showed shorter incubation periods relative to older sheep. Findings suggest that, for SSBP/1, neuroinvasion may occur from highly innervated peripheral tissues like the skin, with infection and replication facilitated by immature nerves. Whether or not this finding can be extrapolated to the BSE agent is intriguing. A marked difference in incubation periods in young and old sheep following s/c inoculation of the BSE agent will help demonstrate this cell tropism. The paucity of the BSE agent in the LRS following experimental oral exposure of calves to BSE (Somerville *et al.*, 1997; Wells *et al.*, 1998; Wells *et al.*, 2005) suggests that the role of the LRS may not be absolutely essential in BSE neuroinvasion and that there may be an alternative route of TSE neuroinvasion such as nerves supplying lymphoid tissue. Based on this observation, it will be interesting to determine whether or not young and old sheep display similar incubation periods following oral exposure to strains other than BSE. Whether or not the network of nerve fibres in PP nodules is detectable only at a certain age and/or stage of involution of lymphoid tissue is not known and requires future investigation. Furthermore, if mature nerves are less vulnerable to scrapie infection in sheep (as in mice),

neuroinvasion by the TSE agent may be either delayed or absent following oral inoculation and this may help to explain why older sheep tend to develop the disease less readily. It must be remembered, however, that the relative importance of different routes of spread of the TSE agent within the body is determined not only by agent-dependent factors but also by a number of host-dependent factors and, therefore, generalizations from an experimental model to natural disease across a species barrier may not be appropriate.

In this study, sheep infected with the BSE agent generally had an increased risk of becoming TSE animals and also developed disease after shorter incubation periods (Chapter 2). Previous findings have established that the vagus and splanchnic nerves are involved in routing of the 263K scrapie strain from the gastrointestinal tract to the brain and spinal cord in hamsters (Beekes and McBride, 2000). This pattern of spread is very similar to that reported for sheep with natural scrapie (Van Keulen *et al.*, 2000). However, it is by no means certain that these neural pathways are ubiquitous to all TSE strains or are the only avenues involved in peripheral spread. While TSE infection of the LRS is necessary for neuroinvasion in many TSE diseases, the possibility remains that the BSE agent may utilise other, hitherto unknown, routes to facilitate its more rapid spread to the CNS following peripheral exposure resulting in quicker onset of disease. This is based on the observation that in natural cases of BSE, TSE infectivity has not been detected in lymph nodes or spleen, and PrP^{Sc} was not found in the distal ileum in cattle (Terry *et al.*, 2003). This is in sharp contrast to what is observed during scrapie in sheep, hamsters and mice, whereby the agent is easily detectable in spleen, tonsils and other lymphatic tissues (Andréoletti *et al.*, 2000; Schreuder *et al.*, 1998) even during the preclinical stage. Furthermore, in contrast to cattle with BSE, most sheep infected orally with BSE-infected brain tissue showed disease-specific PrP accumulations in the LRS (Jeffrey *et al.*, 2001b). However, following experimental oral exposure of calves to the BSE agent, BSE infectivity was found in the distal ileum and a single sample of tonsil, but not in spleen or lymph nodes (Somerville *et al.*, 1997; Wells *et al.*, 1998; Wells *et al.*, 2005). In cattle, the absence of infectivity in the LRS in natural cases of BSE suggests that alternate routes of neuroinvasion may exist and could involve direct infection of the nervous system. One may claim that experimental studies with cattle following oral exposure to the BSE agent may not be representative of the natural situation of GALT in cattle. In Chapter 4, it is assumed that TSE infectivity occurs in PPs of cattle and this may require justification. A recent study using an experimental model of TME provides support for TSE neuroinvasion via cranial nerves from highly innervated peripheral tissues like the tongue, in the absence of LRS infection in natural TSEs of livestock (Bartz *et al.*,

2005). However, absence of infectivity in lymphoid organs may also be the result of low levels of exposure to the BSE agent in natural cases of the disease, and BSE infectivity being present in these tissues at concentrations low enough to be undetectable. One study used transgenic mice over-expressing bovine PrP^C (Tgbov XV mice) and provided further evidence that the pathogenesis of BSE in cattle is fundamentally different from that of scrapie in sheep and mice, and that the spread of BSE infectivity in cattle may be largely neurotropic (Buschmann and Groschup, 2005). Tgbov XV mice were challenged with various tissues from cattle with end-stage clinical BSE and although BSE infectivity was not found in the spleen or mesenteric lymph nodes of terminally BSE-diseased cattle, infectivity was detected in the PPs of the distal ileum, which are the most likely sites of entry for the BSE agent. The latter study indicates that Tgbov XV mice serve not only as a more sensitive mouse model for BSE infectivity, but also that BSE infectivity propagates poorly in bovine PPs and from there, spread to the CNS via nonlymphatic tissue, such as those of the enteric nervous system and the PNS (Buschmann and Groschup, 2005). Several authors have suggested that variations in PrP^C molecular features in peripheral lymphoid organs and more specifically on FDCs may not be detectable by commonly used methods and could be related to the absence of detectable infectivity in the LRS in BSE-affected cattle (Flechsig *et al.*, 2000; Thielen *et al.*, 2001).

Dose and route of exposure were analysed as a combined category in this study (Chapter 2). Hence, effects of these individual factors were not evaluated. However, in general, the oral route resulted in longer incubation periods and was less efficient in transmitting scrapie compared to other routes of infection. This is in accordance with findings from other studies involving the transmission of BSE to Cheviot sheep via similar routes (Goldmann *et al.*, 1994). Experimental evidence in hamsters and mice also indicates that the incubation period is a function of the route of infection and the infective dose. In general, the incubation period is lengthened (McLean and Bostock, 2000) and the attack rate decreased (Baier *et al.*, 2003) when a smaller dose of agent is given. An increase in scrapie incidence in sheep and a decline in age at which disease ensues have been reported within infected flocks, and have been attributed to increasing levels of environmental contamination (Foster and Dickinson, 1989; Sigurdarson, 1991). Exposure to a high dose of agent may result in direct spread to peripheral nerves without prior amplification of infectivity in lymphoreticular tissues resulting, most likely, in overt clinical signs of disease. Oral inoculation with increased dilutions of scrapie-infected brain homogenate resulted in subclinical infections in hamsters that remained as apparently healthy survivors at 520 days post-infection (Baier *et al.*, 2003).

It is likely from these observations that the importance of lymphoreticular amplification may vary with the route of inoculation, infective dose and perhaps the strain of infecting agent thus determining to some extent whether or not an individual may develop clinical signs of disease.

The presence of PrP-expressing FDCs in lymphoid tissue of newborn sheep raises the possibility that sheep could well be susceptible to TSE infection before birth and that transmission of the agent may occur. Studies in sheep have shown that FDCs in lymph nodes can be detected as early as day 120-130 of gestation and acquire mature morphology at about 42 days post-partum (Halleraker *et al.*, 1994). In humans, FDC networks in spleen have been detected by 22 weeks of gestation and exhibit mature lymphoid morphology at one year after birth (Timens *et al.*, 1987). Because FDCs are present prenatally in foetal humans and sheep, sheep scrapie models may serve as a potentially useful model for investigating maternal transmission of vCJD and BSE in humans (Ierna, 2006). Although claims of vertical transmission have been reiterated frequently in the literature, re-examination of the source data reveals that these data are extremely sparse, non-reproducible and probably subject to ascertainment bias. Amniotic fluid and caruncle samples that were tested using a sensitive TgBov XV mouse bioassay were found to be free of detectable BSE infectivity providing further evidence that maternal transmission from cow to calf probably does not play an important role in the epidemiology of BSE (Buschmann and Groschup, 2005). However, this does not rule out the possibility that young lambs are born with relatively developed lymphoreticular responses, which makes them highly susceptible to TSE infection upon exposure to their surrounding environment and may explain the presence of PrP^{Sc} in lymphoid tissues, including ileal PPs, in animals as young as 5 and 9 months old (Van Keulen *et al.*, 2000); This thesis).

There is ample evidence that within lymphoreticular tissue, FDCs play a potentially important role in amplification and spread of the TSE agent in some peripheral models of infection (Brown *et al.*, 1999; Mabbott *et al.*, 2000; McBride *et al.*, 1992). After oral inoculation with TSE-infected material, PrP^{Sc} is initially detected on FDCs in PPs of mice (Beekes and McBride, 2000) and sheep (van Keulen *et al.*, 1996). PrP^{Sc} is also found in lymphoid follicles of PPs following oral infection of non-human primates with BSE-infected material (Bons *et al.*, 1999). Mice with genetic defects resulting in reduced numbers of PPs are resistant to oral challenge with the TSE agent (Prinz *et al.*, 2003) and provide additional evidence supporting a key role of these structures in oral TSE infection. Golovkina *et al.* (1999) investigated the organogenic role of B lymphocytes in mucosal immunity and found

that the development of PPs, FAE and M cells was impaired in mice that had no B cells, and that transgenic expression of membrane-bound immunoglobulin M restored B cells and FAE development (Golovkina *et al.*, 1999). Since members of the TNF-R family have been implicated in organogenesis of lymphoid tissue, including GALT (Debard *et al.*, 1999), they are likely to be involved in the B cell-dependent development of FAE, similar to B-cell dependent generation of FDCs (Fu *et al.*, 1998). Therefore, care should be taken when interpreting studies that implicate indirect effects of B-cells on solely FDCs in peripheral scrapie pathogenesis.

Experimental mouse scrapie models have shown that mature FDCs, expressing the host prion protein (PrP^C), are critical for replication of infection in lymphoid tissues and subsequent neuroinvasion in some (Brown *et al.*, 1999; Mabbott *et al.*, 2000; McBride *et al.*, 1992) but not all (Shlomchik *et al.*, 2001) i/p models of infection. The overwhelming body of evidence in support of the role of FDCs in TSE pathogenesis in adult mice has raised the suspicion that neonatal mice are perhaps less susceptible to scrapie due to a lack of PrP^C-expressing FDCs. Previous studies in splenic tissue of mice have detected PrP^C-expressing FDCs as early as 10 and 14 days post-partum using the FDC-M1 and FDC-M2 markers, respectively (Ierna, 2001). Although this coincides with the time at which neonates appear to acquire full adult susceptibility to scrapie, animals were inoculated intraperitoneally and results of this study may be less relevant to oral pathogenesis (Ierna, 2001). The majority of susceptibility studies have used the i/p and s/c routes of infection in mice. Although age-dependent susceptibility to peripheral scrapie infection in mice may offer a framework with which to investigate the reasons for the current age distribution of vCJD cases, scrapie is not a natural disease of these animals. Furthermore, the role of the spleen and of FDCs in neuron transfer of TSE infectivity following oral infection is unclear, since, although splenectomy prolongs the incubation period of scrapie in animals infected intraperitoneally (Fraser and Dickinson, 1970), it has no effect after oral or intragastric inoculation (Kimberlin and Walker, 1989a). Hence, i/p inoculation in mice is a potentially misleading model of natural oral infection, and the role of FDCs after oral infection with the TSE agent remains to be settled. This makes it difficult to discuss results in relation to susceptibility studies that have used i/p routes of infection.

In an attempt to partly address this issue, PP development and co-localization with PrP was investigated. In mice, PrP^C-expressing FDCs were detected as early as 7 days post-partum. It will be interesting to determine whether neonatal mice, challenged orally, develop full adult

susceptibility to scrapie at around 7 days after birth. If mice are found to be more susceptible to scrapie around this period, then this may be attributed to the development of LRS and may imply that neonatal mice are less able to support scrapie replication in PPs. However, it is important to note that different scrapie strains appear to target different cell types to support their replication, for example, the RML scrapie isolate, unlike the ME7 scrapie strain, may require both PrP-expressing FDCs and lymphocytes (Brown *et al.*, 1999; Prinz *et al.*, 2003), and neuroinvasion by some strains, like the DY TME agent, may not require agent replication in the LRS (Bartz *et al.*, 2005). Further complexity arises because replication of infectivity in lymphoid organs may be more extensive in some species than in others (Baldauf *et al.*, 1997).

In the present study, double immunofluorescence indicated co-localization of PrP and FDCs in PP tissue. In mice, co-localization occurred to a lesser extent compared to results obtained in previous studies that examined spleen tissue (Brown *et al.*, 1999; Ierna, 2001; Ritchie *et al.*, 1999), and may suggest that other cells, in addition to FDCs, may be involved in the replication of TSE infectivity in PPs follwing oral exposure. This issue may be addressed by using other markers to investigate the co-localization of PrP and leukocyte populations, including macrophage and dendritic cell lineages in extra-nodular tissue. Results from previous studies exclude the idea that FDCs (or B cells) are absolutely necessary for infection (including oral infection) and spread to the brain and suggest that other cell types, including macrophages or dendritic cells, may play a more important role in TSE pathogenesis than is generally appreciated (Shlomchik *et al.*, 2001). In some cases, neuroinvasion can occur following oral exposure in the absence of significant lymphoreticular involvement. However, it remains unknown whether an exclusively neural or mixed lymphoreticular-neural pathway is involved in oral TSE infection taking into account strain differences.

Although it is important to investigate the cells that express PrP^C in order to determine where the agent most likely replicates and spreads, competency of expression does not necessarily indicate that a cell is receptive to infection. Events that lead to an increased expression of PrP^C in peripheral compartments, such as gastrointestinal inflammation (Pammer *et al.*, 2000) may also lead to a greater efficiency of neuroinvasion by TSE agent that enters via the oral route. It has also been shown that in inflamed human ileal mucosa, M cells are damaged and increased in number, leading to interruption of the gut epithelial lining and allowing luminal contents access to the underlying lymphoid tissue (Cuvelier *et al.*, 1993). This

scenario fits well with results showing shortened incubation periods in parasitized sheep (Elsen *et al.*, 1999) and in animals with peripheral inflammation (Thackray *et al.*, 2002) and suggests that the physiological state of the gastrointestinal tract may be a contributing factor to susceptibility to TSEs. In this thesis, ileal PP tissue was collected and quantified from sheep with no clinical signs or pathological evidence of intestinal disease (Chapter 3). However, it will be interesting to investigate further oral TSE pathogenesis for example in mice with gastrointestinal inflammation.

Many gaps exist in our knowledge and understanding of the structural elements involved in oral TSE pathogenesis and most studies raise at least as many questions as they answer. Recently, new GALT, referred to as ILFs, has been described in the murine small intestine (Hamada *et al.*, 2002). ILFs occur as tiny lymphoid aggregations aligned along the antimesenteric wall of the mucosa. ILFs are structurally and functionally similar to the follicular units that comprise PP and are believed to be an equivalent, failsafe and/or complementary system to PP for the maintenance of intestinal immune surveillance. The findings of Hamada and colleagues (2002) are important because researchers have been investigating the role of PPs in the pathogenesis of TSEs using various manipulated laboratory mice, including mice that lack PP but possess ILFs. If ILFs serve as a failsafe system to PPs, it is not surprising that in the absence of PPs, neuroinvasion by the TSE agent following oral infection may occur from FDCs in mature ILFs, rendering mice deficient in PPs to be just as susceptible to TSE infection as their controls. Transgenic mice lacking FDCs and/or ILFs will serve as a good model for ascertaining the role of FDCs in future oral TSE pathogenesis studies. However, because the histogenesis and the molecular characteristics of FDCs remain ill-defined, a definitive assessment of the contribution of these cells to TSE pathogenesis continues to be problematic (Aguzzi *et al.*, 2003). Although FDCs express S-100 proteins, as well as complement receptors 2 (CD35) and 4 (identical to the marker FDC-M2), these markers are also expressed by other cell types including those residing within lymphoid organs (Bofill *et al.*, 2000). The FDC-M1 marker, although it seems to be more specific, also stains tingible body macrophages, which are most likely of haematopoietic origin. For this reason, the FDC-M1 marker was not used in this study.

The young age distribution of natural cases of TSEs, including vCJD, BSE and scrapie may reflect age-related exposure, age-dependent incubation periods or age-related susceptibility. Results of this study (Chapter 2; Section 2.3.1) suggest that sheep facing infection early in life seemed more likely to be scrapie positive animals compared to animals facing infection

at older ages, providing further evidence for greater susceptibility to natural TSE infection in young individuals. However, because natural scrapie was first recognised in the NPU flock in 1968 and experiments were carried out from 1960 to 2002 (as well as the unavailability of a preclinical test for the disease) the 'age at exposure' was not always known and the study, therefore, does not provide sound evidence to support the hypothesis of age-dependent susceptibility to TSEs. Nonetheless, similar observations have been made using a model of population dynamics of scrapie where the authors suggest that the effect of age at first exposure may be explained by a higher degree of susceptibility per se of younger, compared with older animals (Matthews et al., 2001). Diaz et al. (2005) have also found that sheep exposed to infection for the first time early in life seemed more likely to show signs of scrapie than animals exposed to infection for the first time at older ages. For the human population, analysis has provided evidence that exposure alone could not explain the young age of vCJD cases seen in the UK and decreasing age-related susceptibility had to be assumed to reproduce the characteristics of the age distribution of vCJD cases (Boelle et al., 2004).

In this present study, younger sheep appeared to have shorter incubation periods when infected by the s/c route only (Chapter 2; Figure 2.3); no similar effect of the oral route was observed. However, plots of the stratified fit and Kaplan-Meier estimates (used to help determine whether young and old animals had equal survivor functions for the different routes of inoculation) provide non-parametric estimates and do not take into account the confounding effect of the other variables. In addition, the sample size for oral inoculation was small with the majority of inoculations (32/37) comprising the BSE agent (as opposed to s/c inoculations where the SSBP/1 agent was used in the majority of cases (408/420)). Different agents may also behave differently (for example, different TSE strains may demonstrate significant differences in cellular tropism) depending on the route of inoculation and the amount of inoculum used. It has been shown that, following inoculation through scarified skin, a functional immune system is critical for the transport of scrapie to the brain as SCID mice were found to be refractory to infection (Mohan et al., 2004). This is consistent with the hypothesis that scrapie infectivity is unlikely to reach the CNS from the skin by direct capture of infectivity by nerves within the skin or by direct transport via the bloodstream (Mohan et al., 2004). Hence, any conclusive statements regarding the observed effect of the oral route and associated lymphoid tissue in the gut in the two age groups of animals would therefore, be 'premature'. A major drawback of this study is that animals exposed at older ages may have been culled or died for non-scrapie reasons (intercurrent

deaths); this may have biased the estimation of the hazard assigned to older animals (Chapter 2) making the reliability of these results questionable.

As mentioned previously, oral infection is the most likely route of natural exposure and PPs are the most probable sites for intestinal uptake of prions following exposure. It is becoming clear that there is great heterogeneity for susceptibility to natural TSE infection, and the association made between PP tissue development and susceptibility to disease suggest that the amount of GALT may represent an additional factor (Chapter 4). Biological processes involved in the maturation of GALT, more specifically PP tissue, may contribute to higher susceptibility to TSE infection in young sheep. This possibility was explored using relatively crude measures of lymphoid tissue development in sheep (Chapters 3 and 4), cattle and humans (Chapter 4) and an association between PP development and susceptibility to natural TSE infection was established. (Alternative measures in PP development may be at least as appropriate (for example, in sheep, counts of functionally mature FDCs, but see below)). Age-related changes in PP development appear less obvious in humans compared to that observed in sheep and cattle (Chapter 4; Figure 4.2). For humans, anatomical data was obtained from Cornes (1965). Cornes used different criteria for defining a PP. He counted patches throughout the entire small intestine, and defined a patch as comprising five or more follicles. It is possible that Cornes may have underestimated PP counts and may have assumed that follicles may function in synergy as an integrated unit rather than each follicle retaining its ability to function independently. Nonetheless, the variation in the number of PPs in the human small intestine in different individuals of various ages (as seen form Figure 4.2 in Chapter 4) is obviously greater than is often appreciated and may influence individual susceptibility to TSE disease (and the occasional occurrence of vCJD in an old individual), which centres on these structures. In addition, for both human and cattle anatomical data, the total weight and total number of PP tissue were used as anatomical measures of PP tissue, respectively, both of which are obviously less accurate measures because of the different length of the small intestine in different sized individuals. In this present study, the percentage of PP tissue and the average number of lymphoid follicles per cm^2 of ileum were used as an anatomical measure of PP tissue in sheep because of the various dimensions of the ileum in different sized animals. Despite the different measures used, however, it appears that the quantitative data on PP development for the three species still reflect the same underlying relationship with age.

The percentage of PP tissue is a relatively crude measure and within a species, the diet, and thus the expected antigenic load in the intestine, the fluidity of the intestinal contents, the

frequency of food intake and the time taken between ingestion and evacuation may be important factors in determining the number of PPs in the intestine (Poskitt et al., 1984). This present study study assumes that both measures of PP development and estimates of age-susceptibility are representative of each host species in general and not just the specific populations examined. In addition, it is assumed that the associations investigated have not been distorted by other factors (like antigenic load and frequency of food intake) which might influence PP development and/or susceptibility to TSEs. Given these caveats, it is nonetheless striking that an association between PP development and susceptibility to TSE is evident not just in one species but in three host species (sheep, cattle and humans) with different relationships between these variables and age.

One may argue that in this study (Chapter 4) PPs may not necessarily (and accurately) represent structures capable of accumulating and replicating the TSE agent, and that the number of *functional* FDCs in young and old sheep for example, may be the reason for the difference in susceptibility to TSE infection. However, staining for FDCs (using the D62 antibody) was found to be less diffuse and less intense in old sheep (over 18 months) compared to younger animals (Chapter 5, Figures 5.7 and 5.8) and perhaps may be an indication of the degree of involution and functional activity of lymphoid nodules and their associated follicles. Age-related declines in humoral and cellular components of the immune system have been demonstrated in both *in vitro* and *in vivo* animal and human studies (Burns and Leventhal, 2000). For example, in old mice of about 23 months, FDCs appeared to be functionally and structurally impaired compared with younger animals; the cells appeared atrophic and only a small fraction of antigen transport sites developed (Szakal *et al.*, 1992). It is therefore possible that senescence of immune system function may have a similar effect on TSEs as has been observed due to dedifferentiation of FDCs in mice (Mabbott *et al.*, 2003). Nevertheless, in this present study, the precise role of FDCs was not central in demonstrating age-dependent susceptibility in sheep, cattle and humans (Chapter 4), and all that was required was merely uptake of material, including prion proteins, at PPs in the ileum as well as in other parts of the gut. As discussed later in this chapter, cells other than FDCs (including M cells and dendritic cells) that reside in PPs and/or bear a close association to these lymphoid structures may well play an important role in the pathogenesis of naturally acquired TSE infection. It therefore appears more reasonable to investigate PPs as an entity rather than limiting the epidemiological study (Chapter 4) to only one cell type. What is important to remember is that similar correlations (between PP development and risk of TSE infection) were found in different species with different patterns of PP development and different age-susceptibility relationships (Chapter 4). Had the study (Chapter 4) simply

investigated PP development and scrapie susceptibility in sheep only, a correlation may have been found but, at least theoretically, other factors could have been involved. The comparative approach adopted in this study (Chapter 4) is a well recognised and useful tool for investigating such relationships (Woolhouse et al., 1991; Grassly et al., 2005) particularly since there is no feasible way of determining this experimentally (as one would need to manipulate the age-PP relationship) and experimentation in humans is not possible.

In conclusion, whether the age of vCJD cases is increasing and, if not, whether it should be expected to do so will depend upon an improved understanding of the age-susceptibility relationship of the naturally occurring TSEs which will be of immense value in interpreting current epidemiological trends and making projections for the future. This thesis seeks to explain an epidemiological pattern – the relationship between age and susceptibility to natural TSE infection - and suggests that age at exposure to the TSE agent and development of PP tissue play a potentially important role in determining the incubation period of disease and whether an individual develops disease, and may help to explain the age distribution of the naturally acquired TSEs. The work in this thesis provides a framework for future oral TSE pathogenesis and age susceptibility studies and brings to light the developmental differences in PrP-expressing FDCs in PPs that exist between the species. Further investigations are clearly needed to further understand these pathways and to evaluate the interactive role of agent and host factors and age specificity of disease risk.

References

Abe, K. and Ito, T. (1978). Qualitative and quantitative morphologic study of Peyer's patches of the mouse after neonatal thymectomy and hydrocortisone injection. *Am J Anat,* **151,** 227-237.

Adams, D. H. and Field, E. J. (1967). A plasma lactic dehydrogenase-elevating virus associated with scrapie-infected mice. *J Gen Virol,* **1,** 449-454.

Aguzzi, A., Heppner, F. L., Heikenwalder, M., Prinz, M., Mertz, K., Seeger, H. and Glatzel, M. (2003). Immune system and peripheral nerves in propagation of prions to CNS. *Br Med Bull,* **66,** 141-159.

Aguzzi, A. and Weissmann, C. (1997). Prion research: the next frontiers. *Nature,* **389,** 795-798.

Aiken, J. M. and Marsh, R. F. (1990). The search for scrapie agent nucleic acid. *Microbiol Rev,* **54,** 242-246.

Alper, T. (1992). New insight into the nature of scrapie from old radiation results. *BJR Suppl,* **24,** 1-5.

Alper, T., Haig, D. A. and Clarke, M. C. (1978). The scrapie agent: evidence against its dependence for replication on intrinsic nucleic acid. *J Gen Virol,* **41,** 503-516.

Anderson, R. M., Donnelly, C. A., Ferguson, N. M., Woolhouse, M. E., Watt, C. J., Udy, H. J., MaWhinney, S., Dunstan, S. P., Southwood, T. R., Wilesmith, J. W., Ryan, J. B., Hoinville, L. J., Hillerton, J. E., Austin, A. R. and Wells, G. A. (1996). Transmission dynamics and epidemiology of BSE in British cattle. *Nature,* **382,** 779-788.

Andréoletti, O., Berthon, P., Levavasseur, E., Marc, D., Lantier, F., Monks, E., Elsen, J. M. and Schelcher, F. (2002a). Phenotyping of protein-prion (PrPsc)-accumulating cells in lymphoid and neural tissues of naturally scrapie-affected sheep by double-labeling immunohistochemistry. *J Histochem Cytochem,* **50,** 1357-1370.

Andréoletti, O., Berthon, P., Marc, D., Sarradin, P., Grosclaude, J., van Keulen, L., Schelcher, F., Elsen, J.-M. and Lantier, F. (2000). Early accumulation of PrP(Sc) in gut-associated lymphoid and nervous tissues of susceptible sheep from a Romanov flock with natural scrapie. *J Virol,* **81,** 3115-3126.

Andréoletti, O., Lacroux, C., Chabert, A., Monnereau, L., Tabouret, G., Lantier, F., Berthon, P., Eychenne, F., Lafond-Benestad, S., Elsen, J. M. and Schelcher, F. (2002b). PrP(Sc) accumulation in placentas of ewes exposed to natural scrapie: influence of foetal PrP genotype and effect on ewe-to-lamb transmission. *J Gen Virol,* **83,** 2607-2616.

Andrews, N. J., Farrington, C. P., Ward, H. J., Cousens, S. N., Smith, P. G., Molesworth, A. M., Knight, R. S., Ironside, J. W. and Will, R. G. (2003). Deaths from variant Creutzfeldt-Jakob disease in the UK. *Lancet,* **361,** 751-752.

Archer, O. K., Sutherland, D. E. and Good, R. A. (1963). Appendix of the rabbit: A homologue of the bursa in the chicken? *Nature,* **200,** 337-339.

Arnold, M. E. and Wilesmith, J. W. (2004). Estimation of the age-dependent risk of infection to BSE of dairy cattle in Great Britain. *Prev Vet Med,* **66,** 35-47.

Asante, E. A., Linehan, J. M., Desbruslais, M., Joiner, S., Gowland, I., Wood, A. L., Welch, J., Hill, A. F., Lloyd, S. E., Wadsworth, J. D. and Collinge, J. (2002). BSE prions propagate as either variant CJD-like or sporadic CJD-like prion strains in transgenic mice expressing human prion protein. *Embo J,* **21,** 6358-6366.

Axon, A. T., Beilenhoff, U., Bramble, M. G., Ghosh, S., Kruse, A., McDonnell, G. E., Neumann, C., Rey, J. F. and Spencer, K. (2001). Variant Creutzfeldt-Jakob disease (vCJD) and gastrointestinal endoscopy. *Endoscopy,* **33,** 1070-1080.

Baier, M., Norley, S., Schultz, J., Burwinkel, M., Schwarz, A. and Riemer, C. (2003). Prion diseases: infectious and lethal doses following oral challenge. *J Gen Virol,* **84,** 1927-1929.

Baker, H. E., Poulter, M., Crow, T. J., Frith, C. D., Lofthouse, R. and Ridley, R. M. (1991). Aminoacid polymorphism in human prion protein and age at death in inherited prion disease. *Lancet,* **337,** 1286.

Baker, H. F., Ridley, R. M., Wells, G. A. and Ironside, J. W. (1998). Prion protein immunohistochemical staining in the brains of monkeys with transmissible spongiform encephalopathy. *Neuropathol Appl Neurobiol,* **24,** 476-486.

Baldauf, E., Beekes, M. and Diringer, H. (1997). Evidence for an alternative direct route of access for the scrapie agent to the brain bypassing the spinal cord. *J Gen Virol,* **78,** 1187-1197.

Balemba, O. B., Grondahl, M. L., Mbassa, G. K., Semuguruka, W. D., Hay-Smith, A., Skadhauge, E. and Dantzer, V. (1998). The organisation of the enteric nervous system in the submucous and mucous layers of the small intestine of the pig studied by VIP and neurofilament protein immunohistochemistry. *J Anat,* **192,** 257-267.

Balogh, P., Aydar, Y., Tew, J. G. and Szakal, A. K. (2001). Ontogeny of the follicular dendritic cell phenotype and function in the postnatal murine spleen. *Cell Immunol,* **214,** 45-53.

Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y. J., Pulendran, B. and Palucka, K. (2000). Immunobiology of dendritic cells. *Annu Rev Immunol,* **18,** 767-811.

Bartz, J. C., Dejoia, C., Tucker, T., Kincaid, A. E. and Bessen, R. A. (2005). Extraneural prion neuroinvasion without lymphoreticular system infection. *J Virol,* **79,** 11858-11863.

Bartz, J. C., Kincaid, A. E. and Bessen, R. A. (2003). Rapid prion neuroinvasion following tongue infection. *J Virol,* **77,** 583-591.

Basler, K., Oesch, B., Scott, M., Westaway, D., Walchli, M., Groth, D. F., McKinley, M. P., Prusiner, S. B. and Weissmann, C. (1986). Scrapie and cellular PrP isoforms are encoded by the same chromosomal gene. *Cell,* **46,** 417-428.

Baylis, M., Chihota, C., Stevenson, E., Goldmann, W., Smith, A., Sivam, K., Tongue, S. and Gravenor, M. B. (2004). Risk of scrapie in British sheep of different prion protein genotype. *J Gen Virol,* **85,** 2735-2740.

Baylis, M. and Goldmann, W. (2004). The genetics of scrapie in sheep and goats. *Curr Mol Med,* **4,** 385-396.

Baylis, M., Goldmann, W., Houston, F., Cairns, D., Chong, A., Ross, A., Smith, A., Hunter, N. and McLean, A. R. (2002). Scrapie epidemic in a fully PrP-genotyped sheep flock. *J Gen Virol,* **83,** 2907-2914.

Beekes, M. and McBride, P. A. (2000). Early accumulation of pathological PrP in the enteric nervous system and gut-associated lymphoid tissue of hamsters orally infected with scrapie. *Neurosci Lett,* **278,** 181-184.

Befus, A. D., Johnston, N., Leslie, G. A. and Bienenstock, J. (1980). Gut-associated lymphoid tissue in the chicken. I. Morphology, ontogeny, and some functional characteristics of Peyer's patches. *J Immunol,* **125,** 2626-2632.

Belt, P. B., Muileman, I. H., Schreuder, B. E., Bos-de Ruijter, J., Gielkens, A. L. and Smits, M. A. (1995). Identification of five allelic variants of the sheep PrP gene and their association with natural scrapie. *J Gen Virol,* **76,** 509-517.

Bendheim, P. E., Barry, R. A., DeArmond, S. J., Stites, D. P. and Prusiner, S. B. (1984). Antibodies to a scrapie prion protein. *Nature,* **310,** 418-421.

Bendheim, P. E., Brown, H. R., Rudelli, R. D., Scala, L. J., Goller, N. L., Wen, G. Y., Kascsak, R. J., Cashman, N. R. and Bolton, D. C. (1992). Nearly ubiquitous tissue distribution of the scrapie agent precursor protein. *Neurology,* **42,** 149-156.

Bennett, A. D., Birkett, C. R. and Bostock, C. J. (1992). Molecular biology of scrapie-like agents. *Rev Sci Tech,* **11,** 569-603.

Bland, J. M. and Altman, D. G. (1986). Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet,* **1,** 307-310.

Blättler, T., Brandner, S., Raeber, A. J., Klein, M. A., Voigtlander, T., Weissmann, C. and Aguzzi, A. (1997). PrP-expressing tissue required for transfer of scrapie infectivity from spleen to brain. *Nature,* **389,** 69-73.

Boëlle, P.-Y., Cesbron, J.-Y. and Valleron, A.-J. (2004). Epidemiological evidence of higher susceptibility to vCJD in the young. *BMC Infect Dis,* **4,** 26-32.

Bofill, M., Akbar, A. N. and Amlot, P. L. (2000). Follicular dendritic cells share a membrane-bound protein with fibroblasts. *J Pathol,* **191,** 217-226.

Bolton, D. C., McKinley, M. P. and Prusiner, S. B. (1982). Identification of a protein that purifies with the scrapie prion. *Science,* **218,** 1309-1311.

Bolton, D. C., McKinley, M. P. and Prusiner, S. B. (1984). Molecular characteristics of the major scrapie prion protein. *Biochemistry,* **23,** 5898-5906.

Bons, N., Mestre-Frances, N., Belli, P., Cathala, F., Gajdusek, D. C. and Brown, P. (1999). Natural and experimental oral infection of nonhuman primates by bovine spongiform encephalopathy agents. *Proc Natl Acad Sci U S A,* **96,** 4046-4051.

Bosque, P. J. (2002). Bovine spongiform encephalopathy, chronic wasting disease, scrapie, and the threat to humans from prion disease epizootics. *Curr Neurol Neurosci Rep,* **2,** 488-495.

Bossers, A., Schreuder, B. E., Muileman, I. H., Belt, P. B. and Smits, M. A. (1996). PrP genotype contributes to determining survival times of sheep with natural scrapie. *J Gen Virol,* **77,** 2669-2673.

Brandel, J. P., Preece, M., Brown, P., Croes, E., Laplanche, J. L., Agid, Y., Will, R. and Alperovitch, A. (2003). Distribution of codon 129 genotype in human growth hormone-treated CJD patients in France and the UK. *Lancet,* **362,** 128-130.

Brandner, S., Klein, M. A., Frigg, R., Pekarik, V., Parizek, P., Raeber, A., Glatzel, M., Schwarz, P., Rulicke, T., Weissmann, C. and Aguzzi, A. (2000). Neuroinvasion of prions: insights from mouse models. *Exp Physiol,* **85,** 705-712.

Brandner, S., Raeber, A., Sailer, A., Blättler, T., Fischer, M., Weissmann, C. and Aguzzi, A. (1996). Normal host prion protein (PrPC) is required for scrapie spread within the central nervous system. *Proc Natl Acad Sci U S A,* **93,** 13148-13151.

Brown, K. L., Ritchie, D. L., McBride, P. A. and Bruce, M. E. (2000a). Detection of PrP in extraneural tissues. *Microsc Res Tech,* **50,** 40-45.

Brown, K. L., Stewart, K., Ritchie, D. L., Mabbott, N. A., Williams, A., Fraser, H., Morrison, W. I. and Bruce, M. E. (1999). Scrapie replication in lymphoid tissues depends on prion protein-expressing follicular dendritic cells. *Nat Med,* **5,** 1308-1312.

Brown, M. C., Jansen, J. K. and Van Essen, D. (1976). Polyneuronal innervation of skeletal muscle in new-born rats and its elimination during maturation. *J Physiol,* **261,** 387-422.

Brown, P. and Gajdusek, D. C. (1991). The human spongiform encephalopathies: kuru, Creutzfeldt-Jakob disease, and the Gerstmann-Sträussler-Scheinker syndrome. *Curr Top Microbiol Immunol,* **172,** 1-20.

Brown, P., Preece, M., Brandel, J. P., Sato, T., McShane, L., Zerr, I., Fletcher, A., Will, R. G., Pocchiari, M., Cashman, N. R., d'Aignaux, J. H., Cervenakova, L., Fradkin, J., Schonberger, L. B. and Collins, S. J. (2000b). Iatrogenic Creutzfeldt-Jakob disease at the millennium. *Neurology,* **55,** 1075-1081.

Brown, P., Preece, M. A. and Will, R. G. (1992). "Friendly fire" in medicine: hormones, homografts, and Creutzfeldt-Jakob disease. *Lancet,* **340,** 24-27.

Bruce, M., Chree, A., McConnell, I., Foster, J., Pearson, G. and Fraser, H. (1994). Transmission of bovine spongiform encephalopathy and scrapie to mice: strain variation and the species barrier. *Philos Trans R Soc Lond B Biol Sci,* **343,** 405-411.

Bruce, M. E. (1993). Scrapie strain variation and mutation. *Br Med Bull,* **49,** 822-838.

Bruce, M. E. (2003). TSE strain variation. *Br Med Bull,* **66,** 99-108.

Bruce, M. E., Boyle, A., Cousens, S., McConnell, I., Foster, J., Goldmann, W. and Fraser, H. (2002). Strain characterization of natural sheep scrapie and comparison with BSE. *J Gen Virol,* **83,** 695-704.

Bruce, M. E. and Dickinson, A. G. (1987). Biological evidence that scrapie agent has an independent genome. *J Gen Virol,* **68,** 79-89.

Bruce, M. E. and Fraser, H. (1991). Scrapie strain variation and its implications. *Curr Top Microbiol Immunol,* **172,** 125-138.

Bruce, M. E., McBride, P. A. and Farquhar, C. F. (1989). Precise targeting of the pathology of the sialoglycoprotein, PrP, and vacuolar degeneration in mouse scrapie. *Neurosci Lett,* **102,** 1-6.

Bruce, M. E., McConnell, I., Fraser, H. and Dickinson, A. G. (1991). The disease characteristics of different strains of scrapie in Sinc congenic mouse lines: implications for the nature of the agent and host control of pathogenesis. *J Gen Virol,* **72,** 595-603.

Bruce, M. E., McConnell, I., Will, R. G. and Ironside, J. W. (2001). Detection of variant Creutzfeldt-Jakob disease infectivity in extraneural tissues. *Lancet,* **358,** 208-209.

Bruce, M. E., Will, R. G., Ironside, J. W., McConnell, I., Drummond, D., Suttie, A., McCardle, L., Chree, A., Hope, J., Birkett, C., Cousens, S., Fraser, H. and Bostock, C. J. (1997). Transmissions to mice indicate that 'new variant' CJD is caused by the BSE agent. *Nature,* **389,** 498-501.

Brugere-Picoux, J., Adjou, K. and Brugere, H. (2005). Update on transmissible spongiform subacute encephalopathies (TSSE). *Bull Acad Natl Med,* **189,** 389-398.

Büeler, H., Aguzzi, A., Sailer, A., Greiner, R. A., Autenried, P., Aguet, M. and Weissmann, C. (1993). Mice devoid of PrP are resistant to scrapie. *Cell,* **73,** 1339-1347.

Büeler, H., Fischer, M., Lang, Y., Bluethmann, H., Lipp, H. P., DeArmond, S. J., Prusiner, S. B., Aguet, M. and Weissmann, C. (1992). Normal development and behaviour of mice lacking the neuronal cell-surface PrP protein. *Nature,* **356,** 577-582.

Burns, E. A. and Leventhal, E. A. (2000). Aging, immunity, and cancer. *Cancer Control,* **7,** 513-522.

Burthem, J. and Roberts, D. J. (2003). The pathophysiology of variant Creutzfeldt-Jacob disease: the hypotheses behind concerns for blood components and products. *Br J Haematol,* **122,** 3-9.

Buschmann, A. and Groschup, M. H. (2005). Highly bovine spongiform encephalopathy-sensitive transgenic mice confirm the essential restriction of infectivity to the nervous system in clinically diseased cattle. *J Infect Dis,* **192,** 934-942.

Carlens, O. (1928). Studien über das lymphatische gewebe des darmkanals bei einigen haustieren, mit besonderer berücksichtingung der embryonalen entwicklung, der mengenverhaltnisse und der altersinvolution dieses gewebs im dünndarm des rindes. *Z Anat Entwicklungs,* **86,** 393-493.

Carlson, G. A. (1996). Prion strains. *Curr Top Microbiol Immunol,* **207,** 35-47.

Carlson, G. A., Ebeling, C., Yang, S. L., Telling, G., Torchia, M., Groth, D., Westaway, D., DeArmond, S. J. and Prusiner, S. B. (1994). Prion isolate specified allotypic interactions between the cellular and scrapie prion proteins in congenic and transgenic mice. *Proc Natl Acad Sci U S A,* **91,** 5690-5694.

Carlson, G. A., Kingsbury, D. T., Goodman, P. A., Coleman, S., Marshall, S. T., DeArmond, S., Westaway, D. and Prusiner, S. B. (1986). Linkage of prion protein and scrapie incubation time genes. *Cell,* **46,** 503-511.

Carlson, J. R. and Owen, R. L. (1987). Structure and functional role of Peyer's patches. In: *Immunopathology of the Small Intestine*, M. N. Marsh, Ed, John Wiley and Sons Ltd, Chichester, United Kingdom, pp. 22-40.

Carp, R. I., Merz, P. A., Kascsak, R. J., Merz, G. S. and Wisniewski, H. M. (1985). Nature of the scrapie agent: current status of facts and hypotheses. *J Gen Virol,* **66,** 1357-1368.

Cashman, N. R., Loertscher, R., Nalbantoglu, J., Shaw, I., Kascsak, R. J., Bolton, D. C. and Bendheim, P. E. (1990). Cellular isoform of the scrapie agent protein participates in lymphocyte activation. *Cell,* **61,** 185-192.

Cella, M., Sallusto, F. and Lanzavecchia, A. (1997). Origin, maturation and antigen presenting function of dendritic cells. *Curr Opin Immunol,* **9,** 10-16.

Cervenakova, L., Goldfarb, L. G., Garruto, R., Lee, H. S., Gajdusek, D. C. and Brown, P. (1998). Phenotype-genotype studies in kuru: implications for new variant Creutzfeldt-Jakob disease. *Proc Natl Acad Sci U S A,* **95,** 13239-13241.

Chandler, R. L. (1961). Encephalopathy in mice produced by inoculation with scrapie brain material. *Lancet,* **1,** 1378-1379.

Chandler, R. L. and Fisher, J. (1963). Experimental transmission of scrapie to rats. *Lancet,* **41,** 1165.

Chase-Topping, M. E., Kruuk, L. E., Lajous, D., Touzeau, S., Matthews, L., Simm, G., Foster, J. D., Rupp, R., Eychenne, F., Hunter, N., Elsen, J. M. and Woolhouse, M. E. (2005). Genotype-

level variation in lifetime breeding success, litter size and survival of sheep in scrapie-affected flocks. *J Gen Virol,* **86,** 1229-1238.

Chatelain, J., Baron, H., Baille, V., Bourdonnais, A., Delasnerie-Laupretre, N. and Cathala, F. (1986). Study of endemic scrapie in a flock of "Ile de France" sheep. *Eur J Epidemiol,* **2,** 31-35.

Chesebro, B., Race, R., Wehrly, K., Nishio, J., Bloom, M., Lechner, D., Bergstrom, S., Robbins, K., Mayer, L., Keith, J. M. and et al. (1985). Identification of scrapie prion protein-specific mRNA in scrapie-infected and uninfected brain. *Nature,* **315,** 331-333.

Clarke, P. and Ghani, A. C. (2004). Projections of the future course of the primary vCJD epidemic in the UK: inclusion of subclinical infection and the possibility of wider genetic susceptibility. *J R Soc Interface,* **2,** 19-31.

Clouscard, C., Beaudry, P., Elsen, J. M., Milan, D., Dussaucy, M., Bounneau, C., Schelcher, F., Chatelain, J., Launay, J. M. and Laplanche, J. L. (1995). Different allelic effects of the codons 136 and 171 of the prion protein gene in sheep with natural scrapie. *J Gen Virol,* **76,** 2097-2101.

Collinge, J., Palmer, M. S. and Dryden, A. J. (1991). Genetic predisposition to iatrogenic Creutzfeldt-Jakob disease. *Lancet,* **337,** 1441-1442.

Collinge, J., Palmer, M. S., Sidle, K. C., Gowland, I., Medori, R., Ironside, J. and Lantos, P. (1995). Transmission of fatal familial insomnia to laboratory animals. *Lancet,* **346,** 569-570.

Cooper, J. D. and Bird, S. M. (2002). UK dietary exposure to BSE in beef mechanically recovered meat: by birth cohort and gender. *J Cancer Epidemiol and Prev,* **7,** 59-70.

Cooper, M. D. and Lawton, A. R., 3rd. (1973). Cellular aspects of immune deficiency diseases involving B cells. *Adv Exp Med Biol,* **29,** 283-287.

Cornes, J. S. (1965). Number, size, and distribution of Peyer's patches in human small intestine. *Gut,* **6,** 225-233.

Cox, D. R. and Isham, V. (1980). *Point Processes.* Chapman and Hall, London.

Craig, S. W. and Cebra, J. J. (1971). Peyer's patches: an enriched source of precursors for IgA-producing immunocytes in the rabbit. *J Exp Med,* **134,** 188-200.

Cuvelier, C. A., Quatacker, J., Mielants, H., De Vos, M., Veys, E. and Roels, H. (1993). M cells are damaged and increased in number in inflamed human ileal mucosa. *Eur J Morphol,* **31,** 87-91.

DeArmond, S. J. (1993). Overview of the transmissible spongiform encephalopathies: prion protein disorders. *Br Med Bull,* **49,** 725-737.

DeArmond, S. J., Mobley, W. C., DeMott, D. L., Barry, R. A., Beckstead, J. H. and Prusiner, S. B. (1987). Changes in the localization of brain prion proteins during scrapie infection. *Neurology,* **37,** 1271-1280.

Debard, N., Sierro, F. and Kraehenbuhl, J. P. (1999). Development of Peyer's patches, follicle-associated epithelium and M cell: lessons from immunodeficient and knockout mice. *Semin Immunol,* **11,** 183-191.

Defra. (2004). Statistics - incidences of BSE [http://www.defra.gov.uk/animalh/bse/statistics/incidence.html]. Accessed on 28/10/04.

Detweiler, L. A. and Baylis, M. (2003). The epidemiology of scrapie. *Rev. Sci. Tech. OIE.,* **22,** 121-143.

Diaz, C., Vitezica, Z. G., Rupp, R., Andréoletti, O. and Elsen, J. M. (2005). Polygenic variation and transmission factors involved in the resistance/susceptibility to scrapie in a Romanov flock. *J Gen Virol,* **86,** 849-857.

Dickinson, A. G. (1976). Scrapie in sheep and goats. *Front Biol,* **44,** 209-241.

Dickinson, A. G. and Fraser, H. (1977). Scrapie pathogenesis in inbred mice: an assessment of host control and response involving many strains of agent. In: *Slow Virus Infections of the Central Nervous System*, V. ter Meulen and M. Karz, Eds, Springer-Verlag, New York, pp. 3-14.

Dickinson, A. G. and Meikle, V. M. (1971). Host-genotype and agent effects in scrapie incubation: change in allelic interaction with different strains of agent. *Mol Gen Genet,* **112,** 73-79.

Dickinson, A. G., Meikle, V. M. and Fraser, H. (1968). Identification of a gene which controls the incubation period of some strains of scrapie agent in mice. *J Comp Pathol,* **78,** 293-299.

Dickinson, A. G. and Outram, G. W. (1988). Genetic aspects of unconventional virus infections: the basis of the virino hypothesis. *Ciba Found Symp,* **135,** 63-83.

Dickinson, A. G. and Stamp, J. T. (1969). Experimental scrapie in Cheviot and Suffolk sheep. *J Comp Pathol,* **79,** 23-26.

Dickinson, A. G., Young, G. B., Stamp, J. T. and Renwick, C. C. (1964). A note on the distribution of scrapie in sheep of different ages. *Anim Prod,* **6,** 375-377.

Diener, T. O. (1972). Is the scrapie agent a viroid? *Nat New Biol,* **235,** 218-219.

Diringer, H., Hilmert, H., Simon, D., Werner, E. and Ehlers, B. (1983). Towards purification of the scrapie agent. *Eur J Biochem,* **134,** 555-560.

Diringer, H., Roehmel, J. and Beekes, M. (1998). Effect of repeated oral infection of hamsters with scrapie. *J Gen Virol,* **79,** 609-612.

Dlouhy, S. R., Hsiao, K., Farlow, M. R., Foroud, T., Conneally, P. M., Johnson, P., Prusiner, S. B., Hodes, M. E. and Ghetti, B. (1992). Linkage of the Indiana kindred of Gerstmann-Sträussler-Scheinker disease to the prion protein gene. *Nat Genet,* **1,** 64-67.

Eglin, R. D., Warner, R., Gubbins, S., Sivam, S. K. and Dawson, M. (2005). Frequencies of PrP genotypes in 38 breeds of sheep sampled in the National Scrapie Plan for Great Britain. *Vet Rec,* **156,** 433-437.

Eklund, C. M., Kennedy, R. C. and Hadlow, W. J. (1967). Pathogenesis of scrapie virus infection in the mouse. *J Infect Dis,* **117,** 15-22.

Elsen, J. M., Amigues, Y., Schelcher, F., Ducrocq, V., Andréoletti, O., Eychenne, F., Khang, J. V., Poivey, J. P., Lantier, F. and Laplanche, J. L. (1999). Genetic susceptibility and transmission factors in scrapie: detailed analysis of an epidemic in a closed flock of Romanov. *Arch Virol,* **144,** 431-445.

Endres, R., Alimzhanov, M. B., Plitz, T., Futterer, A., Kosco-Vilbois, M. H., Nedospasov, S. A., Rajewsky, K. and Pfeffer, K. (1999). Mature follicular dendritic cell networks depend on expression of lymphotoxin beta receptor by radioresistant stromal cells and of lymphotoxin beta and tumor necrosis factor by B cells. *J Exp Med,* **189,** 159-168.

Fang, Y., Xu, C., Fu, Y. X., Holers, V. M. and Molina, H. (1998). Expression of complement receptors 1 and 2 on follicular dendritic cells is necessary for the generation of a strong antigen-specific IgG response. *J Immunol,* **160,** 5273-5279.

Farquhar, C. F., Dornan, J., Somerville, R. A., Tunstall, A. M. and Hope, J. (1994). Effect of Sinc genotype, agent isolate and route of infection on the accumulation of protease-resistant PrP in non-central nervous system tissues during the development of murine scrapie. *J Gen Virol,* **75,** 495-504.

Farquhar, C. F., Somerville, R. A. and Ritchie, L. A. (1989). Post-mortem immunodiagnosis of scrapie and bovine spongiform encephalopathy. *J Virol Methods,* **24,** 215-221.

Faulk, W. P., McCormick, J. N., Goodman, J. R., Yoffey, J. M. and Fudenberg, H. H. (1970). Peyer's patches: morphologic studies. *Cell Immunol,* **1,** 500-520.

Ferguson, N. M., Donnelly, C. A., Woolhouse, M. E. and Anderson, R. M. (1997). The epidemiology of BSE in cattle herds in Great Britain. II. Model construction and analysis of transmission dynamics. *Philos. T. Roy. Soc. B.,* **352,** 803-838.

Field, E. J., Raine, C. S. and Joyce, G. (1967). Scrapie in the rat: an electron-microscope study. I. Amyloid bodies and deposits. *Acta Neuropathol (Berl),* **8,** 47-56.

Fitzsimmons, W. M. and Pattison, I. H. (1968). Unsuccessful attempts to transmit scrapie by nematode parasites. *Res Vet Sci,* **9,** 281-283.

Flechsig, E., Shmerling, D., Hegyi, I., Raeber, A. J., Fischer, M., Cozzio, A., von Mering, C., Aguzzi, A. and Weissmann, C. (2000). Prion protein devoid of the octapeptide repeat region restores susceptibility to scrapie in PrP knockout mice. *Neuron,* **27,** 399-408.

Foote, W. C., Clark, W., Maciulis, A., Call, J. W., Hourrigan, J., Evans, R. C., Marshall, M. R. and de Camp, M. (1993). Prevention of scrapie transmission in sheep, using embryo transfer. *Am J Vet Res,* **54,** 1863-1868.

Foster, J. D. and Dickinson, A. G. (1988a). Genetic control of scrapie in Cheviot and Suffolk sheep. *Vet Rec,* **123,** 159.

Foster, J. D. and Dickinson, A. G. (1988b). The unusual properties of CH1641, a sheep-passaged isolate of scrapie. *Vet Rec,* **123,** 5-8.

Foster, J. D. and Dickinson, A. G. (1989). Age at death from natural scrapie in a flock of Suffolk sheep. *Vet Rec,* **125,** 415-417.

Foster, J. D., Hope, J. and Fraser, H. (1993). Transmission of bovine spongiform encephalopathy to sheep and goats. *Vet Rec,* **133,** 339-341.

Foster, J. D. and Hunter, N. (1991). Partial dominance of the sA allele of the Sip gene for controlling experimental scrapie. *Vet Rec,* **128,** 548-549.

Foster, J. D., Hunter, N., Williams, A., Mylne, M. J., McKelvey, W. A., Hope, J., Fraser, H. and Bostock, C. (1996). Observations on the transmission of scrapie in experiments using embryo transfer. *Vet Rec,* **138,** 559-562.

Foster, J. D., McKelvey, W. A., Mylne, M. J., Williams, A., Hunter, N., Hope, J. and Fraser, H. (1992). Studies on maternal transmission of scrapie in sheep by embryo transfer. *Vet Rec,* **130,** 341-343.

Foster, J. D., Parnham, D., Chong, A., Goldmann, W. and Hunter, N. (2001). Clinical signs, histopathology and genetics of experimental transmission of BSE and natural scrapie to sheep and goats. *Vet Rec,* **148,** 165-171.

Fraser, H., Brown, K. L., Stewart, K., McConnell, I., McBride, P. and Williams, A. (1996). Replication of scrapie in spleens of SCID mice follows reconstitution with wild-type mouse bone marrow. *J Gen Virol,* **77,** 1935-1940.

Fraser, H. and Dickinson, A. G. (1968). The sequential development of the brain lesion of scrapie in three strains of mice. *J Comp Pathol,* **78,** 301-311.

Fraser, H. and Dickinson, A. G. (1970). Pathogenesis of scrapie in the mouse: the role of the spleen. *Nature,* **226,** 462-463.

Fraser, H. and Dickinson, A. G. (1978). Studies of the lymphoreticular system in the pathogenesis of scrapie: the role of spleen and thymus. *J Comp Pathol,* **88,** 563-573.

Fraser, H. and Farquhar, C. F. (1987). Ionising radiation has no influence on scrapie incubation period in mice. *Vet Microbiol,* **13,** 211-223.

Fu, Y. X., Huang, G., Wang, Y. and Chaplin, D. D. (1998). B lymphocytes induce the formation of follicular dendritic cell clusters in a lymphotoxin alpha-dependent fashion. *J Exp Med,* **187,** 1009-1018.

Gajdusek, D. C. (1977). Unconventional viruses and the origin and disappearance of kuru. *Science,* **197,** 943-960.

Gerber, H. A., Morris, B. and Trevella, W. (1986). The role of gut-associated lymphoid tissues in the generation of immunoglobulin-bearing lymphocytes in sheep. *Aust J Exp Biol Med Sci,* **64,** 201-213.

Ghani, A., Ferguson, N., Donnelly, C. and Anderson, R. (2003). Factors determining the pattern of the variant Creutzfeldt-Jakob disease (vCJD) epidemic in the UK. *Proc of of the Roy Soc London B Biol Sci,* **270,** 689-698.

Ghani, A. C., Donnelly, C. A., Ferguson, N. M. and Anderson, R. M. (2000). Assessment of the prevalence of vCJD through testing tonsils and appendices for abnormal prion protein. *Proc Biol Sci,* **267,** 23-29.

Ghani, A. C., Ferguson, N. M., Donnelly, C. A., Hagenaars, T. J. and Anderson, R. M. (1998). Estimation of the number of people incubating variant CJD. *Lancet,* **352,** 1353-1354.

Gibbs, C. J., Jr., Amyx, H. L., Bacote, A., Masters, C. L. and Gajdusek, D. C. (1980). Oral transmission of kuru, Creutzfeldt-Jakob disease, and scrapie to nonhuman primates. *J Infect Dis,* **142,** 205-208.

Gibbs, C. J., Jr., Gajdusek, D. C. and Amyx, H. (1979). Strain variation in the viruses of Creutzfeldt-Jakob disease ad kuru. In: *Slow Transmissible Diseases of the Nervous System,* S. B. Prusiner and W. J. Hadlow, Eds, Academic Press.

Glatzel, M., Heppner, F. L., Albers, K. M. and Aguzzi, A. (2001). Sympathetic innervation of lymphoreticular organs is rate limiting for prion neuroinvasion. *Neuron,* **31,** 25-34.

Gleason, T. H. and Patterson, S. D. (1982). The pathology of Yersinia enterocolitica ileocolitis. *Am J Surg Pathol,* **6,** 347-355.

Goldfarb, L. G. and Brown, P. (1995). The transmissible spongiform encephalopathies. *Annu Rev Med,* **46,** 57-65.

Goldfarb, L. G., Cervenakova, L. and Gajdusek, D. C. (2004). Genetic studies in relation to kuru: an overview. *Curr Mol Med,* **4,** 375-384.

Goldfarb, L. G., Petersen, R. B., Tabaton, M., Brown, P., LeBlanc, A. C., Montagna, P., Cortelli, P., Julien, J., Vital, C., Pendelbury, W. W. and et al. (1992). Fatal familial insomnia and familial Creutzfeldt-Jakob disease: disease phenotype determined by a DNA polymorphism. *Science,* **258,** 806-808.

Goldmann, W., Hunter, N., Martin, T., Dawson, M. and Hope, J. (1991). Different forms of the bovine PrP gene have five or six copies of a short, G-C-rich element within the protein-coding exon. *J Gen Virol,* **72,** 201-204.

Goldmann, W., Hunter, N., Smith, G., Foster, J. and Hope, J. (1994). PrP genotype and agent effects in scrapie: change in allelic interaction with different isolates of agent in sheep, a natural host of scrapie. *J Gen Virol,* **75,** 989-995.

Golovkina, T. V., Shlomchik, M., Hannum, L. and Chervonsky, A. (1999). Organogenic role of B lymphocytes in mucosal immunity. *Science,* **286,** 1965-1968.

González, L., Martin, S., Houston, F. E., Hunter, N., Reid, H. W., Bellworthy, S. J. and Jeffrey, M. (2005). Phenotype of disease-associated PrP accumulation in the brain of bovine spongiform encephalopathy experimentally infected sheep. *J Gen Virol,* **86,** 827-838.

Gordon, W. (1946). Advances in Veterinary Research. *Vet Rec*, **58,** 516-520.

Gore, S. M., Gilks, W. R. and Wilesmith, J. W. (1997). BSE maternal cohort study: explanatory analysis. *Appl Stat,* **46,** 305-320.

Grassly, N. C., Fraser, C. & Garnett, G. P. (2005). Host immunity and synchronized epidemics of syphilis across the United States. *Nature* **433**, 417-21.

Gravenor, M. B., Stallard, N., Curnow, R. and McLean, A. R. (2003). Repeated challenge with prion disease: the risk of infection and impact on incubation period. *Proc Natl Acad Sci U S A,* **100,** 10960-10965.

Griebel, P. J. and Hein, W. R. (1996). Expanding the role of Peyer's patches in B-cell ontogeny. *Immunol Today,* **17,** 30-39.

Griffith, J. S. (1967). Self-replication and scrapie. *Nature,* **215,** 1043-1044.

Gruner, L., Elsen, J. M., Vu Tien Khang, J., Eychenne, F., Caritez, J. C., Jacquiet, P., Andréoletti, O., Sarradin, P., Cortet, J., Richer, N. and Leroux, H. (2004). Nematode parasites and scrapie: experiments in sheep and mice. *Parasitol Res,* **93,** 493-498.

Guiroy, D. C., Wakayama, I., Liberski, P. P. and Gajdusek, D. C. (1994). Relationship of microglia and scrapie amyloid-immunoreactive plaques in kuru, Creutzfeldt-Jakob disease and Gerstmann-Sträussler syndrome. *Acta Neuropathol (Berl),* **87,** 526-530.

Hadlow, W. J., Kennedy, R. C. and Race, R. E. (1982). Natural infection of Suffolk sheep with scrapie virus. *J Infect Dis,* **146,** 657-664.

Halleraker, M., Press, C. M. and Landsverk, T. (1994). Development and cell phenotypes in primary follicles of foetal sheep lymph nodes. *Cell Tissue Res,* **275,** 51-62.

Haltia, M., Kovanen, J., Van Crevel, H., Bots, G. T. and Stefanko, S. (1979). Familial Creutzfeldt-Jakob disease. *J Neurol Sci,* **42,** 381-389.

Hamada, H., Hiroi, T., Nishiyama, Y., Takahashi, H., Masunaga, Y., Hachimura, S., Kaminogawa, S., Takahashi-Iwanaga, H., Iwanaga, T., Kiyono, H., Yamamoto, H. and Ishikawa, H. (2002). Identification of multiple isolated lymphoid follicles on the antimesenteric wall of the mouse small intestine. *J Immunol,* **168,** 57-64.

Hamir, A. N., Kunkle, R. A., Richt, J. A., Miller, J. M., Cutlip, R. C. & Jenny, A. L. (2005). Experimental transmission of sheep scrapie by intracerebral and oral routes to genetically susceptible Suffolk sheep in the United States. *J Vet Diagn Invest* **17**, 3-9.

Head, M. W. and Ironside, J. W. (2005). Mad cows and monkey business: the end of vCJD? *Lancet,* **365,** 730-731.

Healy, A. M., Hannon, D., Morgan, K. L., Weavers, E., Collins, J. D. and Doherty, M. L. (2004). A paired case-control study of risk factors for scrapie in Irish sheep flocks. *Prev Vet Med,* **64,** 73-83.

Heel, K. A., McCauley, R. D., Papadimitriou, J. M. and Hall, J. C. (1997). Review: Peyer's patches. *J Gastroenterol Hepatol,* **12,** 122-136.

Heggebø, R., González, L., Press, C. M., Gunnes, G., Espenes, A. and Jeffrey, M. (2003). Disease-associated PrP in the enteric nervous system of scrapie-affected Suffolk sheep. *J Gen Virol,* **84,** 1327-1338.

Heggebø, R., Press, C. M., Gunnes, G., González, L. and Jeffrey, M. (2002). Distribution and accumulation of PrP in gut-associated and peripheral lymphoid tissue of scrapie-affected Suffolk sheep. *J Gen Virol,* **83,** 479-489.

Heggebø, R., Press, C. M., Gunnes, G., Lie, K. I., Tranulis, M. A., Ulvund, M., Groschup, M. H. and Landsverk, T. (2000). Distribution of prion protein in the ileal Peyer's patch of scrapie-free lambs and lambs naturally and experimentally exposed to the scrapie agent. *J Gen Virol,* **81,** 2327-2337.

Hein, W. R., Dudler, L. and Mackay, C. R. (1989). Surface expression of differentiation antigens on lymphocytes in the ileal and jejunal Peyer's patches of lambs. *Immunology,* **68,** 365-370.

Heisey, D. M. and Joly, D. O. (2004). Age and transmissible spongiform encephalopathies. *Emerg Infect Dis,* **10,** 1164-1165.

Heppner, F. L., Christ, A. D., Klein, M. A., Prinz, M., Fried, M., Kraehenbuhl, J. P. and Aguzzi, A. (2001). Transepithelial prion transport by M cells. *Nat Med,* **7,** 976-977.

Herzog, C., Sales, N., Etchegaray, N., Charbonnier, A., Freire, S., Dormont, D., Deslys, J. P. and Lasmézas, C. I. (2004). Tissue distribution of bovine spongiform encephalopathy agent in primates after intravenous or oral infection. *Lancet,* **363,** 422-428.

Hill, A. F., Butterworth, R. J., Joiner, S., Jackson, G., Rossor, M. N., Thomas, D. J., Frosh, A., Tolley, N., Bell, J. E., Spencer, M., King, A., Al-Sarraj, S., Ironside, J. W., Lantos, P. L. and Collinge, J. (1999). Investigation of variant Creutzfeldt-Jakob disease and other human prion diseases with tonsil biopsy samples. *Lancet,* **353,** 183-189.

Hill, A. F. and Collinge, J. (2003). Subclinical prion infection. *Trends Microbiol,* **11,** 578-584.

Hilton, D. A., Ghani, A. C., Conyers, L., Edwards, P., McCardle, L., Penney, M., Ritchie, D. and Ironside, J. W. (2002). Accumulation of prion protein in tonsil and appendix: review of tissue samples. *Br Med J,* **325,** 633-634.

Hilton, D. A., Ghani, A. C., Conyers, L., Edwards, P., McCardle, L., Ritchie, D., Penney, M., Hegazy, D. and Ironside, J. W. (2004). Prevalence of lymphoreticular prion protein accumulation in UK tissue samples. *J Pathol,* **203,** 733-739.

HogenEsch, H., Housman, J. M. and Felsburg, P. J. (1987). Canine Peyer's patches: macroscopic, light microscopic, scanning electron microscopic and immunohistochemical investigations. *Adv Exp Med Biol,* **216,** 249-256.

Hoinville, L., Webb, C. and Wilesmith, J. (2000a). Surveillance for scrapie. *Vet Rec,* **146,** 507.

Hoinville, L. J., Hoek, A., Gravenor, M. B. and McLean, A. R. (2000b). Descriptive epidemiology of scrapie in Great Britain: results of a postal survey. *Vet Rec,* **146,** 455-461.

Holmes, K. L., Schnizlein, C. T., Perkins, E. H. and Tew, J. G. (1984). The effect of age on antigen retention in lymphoid follicles and in collagenous tissue of mice. *Mech Ageing Dev,* **25,** 243-255.

Horiuchi, M., Yamazaki, N., Ikeda, T., Ishiguro, N. and Shinagawa, M. (1995). A cellular form of prion protein (PrPC) exists in many non-neuronal tissues of sheep. *J Gen Virol,* **76,** 2583-2587.

Horn, G. (2001). Review of the origin of BSE, Defra, London, pp. 1-68.

Hourrigan, J. L., Klingsporn, A., Clark, W. W. and De Camp, M. (1979). Epidemiology of scrapie in the United States. In: *Slow Transmissible Diseases of the Nervous System,* S. B. Prusiner and W. J. Hadlow, Eds, Vol. 1, Academic Press, New York, pp. 331-356.

Houston, E. F. and Gravenor, M. B. (2003). Clinical signs in sheep experimentally infected with scrapie and BSE. *Vet Rec,* **152,** 333-334.

Houston, E. F., Halliday, S. I., Jeffrey, M., Goldmann, W. and Hunter, N. (2002). New Zealand sheep with scrapie-susceptible PrP genotypes succumb to experimental challenge with a sheep-passaged scrapie isolate (SSBP/1). *J Gen Virol,* **83,** 1247-1250.

Houston, F., Goldmann, W., Chong, A., Jeffrey, M., González, L., Foster, J., Parnham, D. and Hunter, N. (2003). Prion diseases: BSE in sheep bred for resistance to infection. *Nature,* **423,** 498.

Hsiao, K., Baker, H. F., Crow, T. J., Poulter, M., Owen, F., Terwilliger, J. D., Westaway, D., Ott, J. and Prusiner, S. B. (1989). Linkage of a prion protein missense variant to Gerstmann-Sträussler syndrome. *Nature,* **338,** 342-345.

Hsiao, K. and Prusiner, S. B. (1991). Molecular genetics and transgenic model of Gertsmann-Sträussler-Scheinker disease. *Alzheimer Dis Assoc Disord,* **5,** 155-162.

Huang, Z., Prusiner, S. B. and Cohen, F. E. (1996). Scrapie prions: a three-dimensional model of an infectious fragment. *Fold Des,* **1,** 13-19.

Huillard d'Aignaux, J. N., Cousens, S. N., Maccario, J., Costagliola, D., Alpers, M. P., Smith, P. G. and Alperovitch, A. (2002). The incubation period of kuru. *Epidemiology,* **13,** 402-408.

Hummel, K. (1935). The structure and development of the lymphatic tissue in the intestine of the albino rat. *Am J Anat,* **57,** 351-376.

Humphrey, J. H., Grennan, D. and Sundaram, V. (1984). The origin of follicular dendritic cells in the mouse and the mechanism of trapping of immune complexes on them. *Eur J Immunol,* **14,** 859-864.

Hunter, G. D. (1972). Membrane hypothesis of scrapie. *J Clin Pathol Suppl (R Coll Pathol),* **6,** 152.

Hunter, N. (1997a). Molecular biology and genetics of scrapie in sheep. In: *The Genetics of Sheep*, L. Piper and A. Ruvinsky, Eds, CAB International, United Kingdom.

Hunter, N. (1997b). PrP genetics in sheep and the applications for scrapie and BSE. *Trends Microbiol,* **5,** 331-334.

Hunter, N. (2003). Scrapie and experimental BSE in sheep. *Br Med Bull,* **66,** 171-183.

Hunter, N. and Cairns, D. (1998). Scrapie-free Merino and Poll Dorset sheep from Australia and New Zealand have normal frequencies of scrapie-susceptible PrP genotypes. *J Gen Virol,* **79,** 2079-2082.

Hunter, N., Foster, J. D., Goldmann, W., Stear, M. J., Hope, J. and Bostock, C. (1996). Natural scrapie in a closed flock of Cheviot sheep occurs only in specific PrP genotypes. *Arch Virol,* **141,** 809-824.

Hunter, N., Foster, J. D. and Hope, J. (1992). Natural scrapie in British sheep: breeds, ages and PrP gene polymorphisms. *Vet Rec,* **130,** 389-392.

Hunter, N., Goldmann, W., Foster, J. D., Cairns, D. and Smith, G. (1997). Natural scrapie and PrP genotype: case-control studies in British sheep. *Vet Rec,* **141,** 137-140.

Hunter, N., Goldmann, W., Marshall, E. and O'Neill, G. (2000). Sheep and goats: natural and experimental TSEs and factors influencing incidence of disease. *Arch Virol Suppl,* **16,** 181-188.

Hunter, N., Goldmann, W., Smith, G. and Hope, J. (1994). Frequencies of PrP gene variants in healthy cattle and cattle with BSE in Scotland. *Vet Rec,* **135,** 400-403.

Hunter, N., Hope, J., McConnell, I. and Dickinson, A. G. (1987). Linkage of the scrapie-associated fibril protein (PrP) gene and Sinc using congenic mice and restriction fragment length polymorphism analysis. *J Gen Virol,* **68,** 2711-2716.

Husband, A. J., Monie, H. J. and Gowans, J. L. (1977). The natural history of the cells producing IgA in the gut. *Ciba Found Symp,* **46,** 29-54.

Ibrahim, M. A., Chain, B. M. and Katz, D. R. (1995). The injured cell: the role of the dendritic cell system as a sentinel receptor pathway. *Immunol Today,* **16,** 181-186.

Ierna, M. (2001). An investigation of scrapie pathogenesis in neonatal mice with special reference to germinal centre maturation, University of Edinburgh, Edinburgh, pp. 29.

Ierna, M., Farquhar, C. F., Outram, G. W. and Bruce, M. E. (2006). Resistance of neonatal mice to scrapie is associated with inefficient infection of the immature spleen. *J Virol,* **80,** 474-482.

Ikeda, T., Horiuchi, M., Ishiguro, N., Muramatsu, Y., Kai-Uwe, G. D. and Shinagawa, M. (1995). Amino acid polymorphisms of PrP with reference to onset of scrapie in Suffolk and Corriedale sheep in Japan. *J Gen Virol,* **76,** 2577-2581.

Imai, Y., Yamakawa, M., Masuda, A., Sato, T. and Kasajima, T. (1986). Function of the follicular dendritic cell in the germinal center of lymphoid follicles. *Histol Histopathol,* **1,** 341-353.

Imai, Y., Yamakawa, M. and Kasajima, T. (1998). The lymphocyte-dendritic cell system. *Histol Histopathol,* **13,** 469-510.

Ironside, J. W. (1996). Human prion diseases. *J Neural Transm Suppl,* **47,** 231-246.

Ironside, J. W. and Head, M. W. (2004). Neuropathology and molecular biology of variant Creutzfeldt-Jakob disease. *Curr Top Microbiol Immunol,* **284,** 133-159.

Ishikawa, H., Saito, H., Suzuki, K., Oida, T. and Kanamori, Y. (1999). New gut associated lymphoid tissue "cryptopatches" breed murine intestinal intraepithelial T cell precursors. *Immunol Res,* **20,** 243-250.

Jeffrey, M., Martin, S., González, L., Ryder, S. J., Bellworthy, S. J. and Jackman, R. (2001a). Differential diagnosis of infections with the bovine spongiform encephalopathy (BSE) and scrapie agents in sheep. *J Comp Pathol,* **125,** 271-284.

Jeffrey, M., McGovern, G., Goodsir, C. M., Brown, K. L. and Bruce, M. E. (2000a). Sites of prion protein accumulation in scrapie-infected mouse spleen revealed by immuno-electron microscopy. *J Pathol,* **191,** 323-332.

Jeffrey, M., McGovern, G., Martin, S., Goodsir, C. M. and Brown, K. L. (2000b). Cellular and sub-cellular localisation of PrP in the lymphoreticular system of mice and sheep. *Arch Virol Suppl,* **16,** 23-38.

Jeffrey, M., Ryder, S., Martin, S., Hawkins, S. A., Terry, L., Berthelin-Baker, C. and Bellworthy, S. J. (2001b). Oral inoculation of sheep with the agent of bovine spongiform encephalopathy (BSE). 1. Onset and distribution of disease-specific PrP accumulation in brain and viscera. *J Comp Pathol,* **124,** 280-289.

Jessen, K. R. and Mirsky, R. (1999). Schwann cells and their precursors emerge as major regulators of nerve development. *Trends Neurosci,* **22,** 402-410.

Kanamori, Y., Ishimaru, K., Nanno, M., Maki, K., Ikuta, K., Nariuchi, H. and Ishikawa, H. (1996). Identification of novel lymphoid tissues in murine intestinal mucosa where clusters of c-kit+ IL-7R+ Thy1+ lympho-hemopoietic progenitors develop. *J Exp Med,* **184,** 1449-1459.

Kaneko, K., Ball, H. L., Wille, H., Zhang, H., Groth, D., Torchia, M., Tremblay, P., Safar, J., Prusiner, S. B., DeArmond, S. J., Baldwin, M. A. and Cohen, F. E. (2000). A synthetic peptide initiates Gerstmann-Sträussler-Scheinker (GSS) disease in transgenic mice. *J Mol Biol,* **295,** 997-1007.

Kao, R. R., Houston, F., Baylis, M., Chihota, C. M., Goldmann, W., Gravenor, M. B., Hunter, N. and McLean, A. R. (2003). Epidemiological implications of the susceptibility to BSE of putatively resistant sheep. *J Gen Virol,* **84,** 3503-3512.

Kapasi, Z. F., Burton, G. F., Shultz, L. D., Tew, J. G. and Szakal, A. K. (1993). Induction of functional follicular dendritic cell development in severe combined immunodeficiency mice. Influence of B and T cells. *J Immunol,* **150,** 2648-2658.

Kapasi, Z. F., Qin, D., Kerr, W. G., Kosco-Vilbois, M. H., Shultz, L. D., Tew, J. G. and Szakal, A. K. (1998). Follicular dendritic cell (FDC) precursors in primary lymphoid tissues. *J Immunol,* **160,** 1078-1084.

Karrer, U., Lopez-Macias, C., Oxenius, A., Odermatt, B., Bachmann, M. F., Kalinke, U., Bluethmann, H., Hengartner, H. and Zinkernagel, R. M. (2000). Antiviral B cell memory in the absence of mature follicular dendritic cell networks and classical germinal centers in TNFR1-/- mice. *J Immunology,* **164,** 768-778.

Kellings, K., Meyer, N., Mirenda, C., Prusiner, S. B. and Riesner, D. (1992). Further analysis of nucleic acids in purified scrapie prion preparations by improved return refocusing gel electrophoresis. *J Gen Virol,* **73,** 1025-1029.

Kelsall, B. L. and Strober, W. (1996). The role of dendritic cells in antigen processing in the Peyer's patch. *Ann N Y Acad Sci,* **778,** 47-54.

Kimberlin, R. and Walker, C. (1989a). Pathogenesis of scrapie in mice after intragastric infection. *Virus Res,* **12,** 213-220.

Kimberlin, R. H., Field, H. J. and Walker, C. A. (1983a). Pathogenesis of mouse scrapie: evidence for spread of infection from central to peripheral nervous system. *J Gen Virol,* **64,** 713-716.

Kimberlin, R. H., Hall, S. M. and Walker, C. A. (1983b). Pathogenesis of mouse scrapie. Evidence for direct neural spread of infection to the CNS after injection of sciatic nerve. *J Neurol Sci,* **61,** 315-325.

Kimberlin, R. H. and Millson, G. C. (1967). Some biochemical aspects of mouse scrapie. *J Comp Pathol,* **77,** 359-366.

Kimberlin, R. H., Walker, C. and Fraser, H. (1989). The genomic identity of different strains of mouse scrapie is expressed in hamsters and preserved on reisolation in mice. *J Gen Virol,* **70,** 2017-2025.

Kimberlin, R. H. and Walker, C. A. (1978). Evidence that the transmission of one source of scrapie agent to hamsters involves separation of agent strains from a mixture. *J Gen Virol,* **39,** 487-496.

Kimberlin, R. H. and Walker, C. A. (1988). Incubation periods in six models of intraperitoneally injected scrapie depend mainly on the dynamics of agent replication within the nervous system and not the lymphoreticular system. *J Gen Virol,* **69,** 2953-2960.

Kimberlin, R. H. and Walker, C. A. (1989b). The role of the spleen in the neuroinvasion of scrapie in mice. *Virus Res,* **12,** 201-211.

Kirkwood, J. K. and Cunningham, A. A. (1994). Epidemiological observations on spongiform encephalopathies in captive wild animals in the British Isles. *Vet Rec,* **135,** 296-303.

Kitamoto, T., Muramoto, T., Mohri, S., Doh-Ura, K. and Tateishi, J. (1991). Abnormal isoform of prion protein accumulates in follicular dendritic cells in mice with Creutzfeldt-Jakob disease. *J Virol,* **65,** 6292-6295.

Kitamoto, T., Ogomori, K., Tateishi, J. and Prusiner, S. B. (1987). Formic acid pretreatment enhances immunostaining of cerebral and systemic amyloids. *Lab Invest,* **57,** 230-236.

Kitamura, D., Roes, J., Kuhn, R. and Rajewsky, K. (1991). A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin mu chain gene. *Nature,* **350,** 423-426.

Klein, M. A., Frigg, R., Flechsig, E., Raeber, A. J., Kalinke, U., Bluethmann, H., Bootz, F., Suter, M., Zinkernagel, R. M. and Aguzzi, A. (1997). A crucial role for B cells in neuroinvasive scrapie. *Nature,* **390,** 687-690.

Klein, M. A., Frigg, R., Raeber, A. J., Flechsig, E., Hegyi, I., Zinkernagel, R. M., Weissmann, C. and Aguzzi, A. (1998). PrP expression in B lymphocytes is not required for prion neuroinvasion. *Nat Med,* **4,** 1429-1433.

Korth, C., Stierli, B., Streit, P., Moser, M., Schaller, O., Fischer, R., Schulz-Schaeffer, W., Kretzschmar, H., Raeber, A., Braun, U., Ehrensperger, F., Hornemann, S., Glockshuber, R., Riek, R., Billeter, M., Wuthrich, K. and Oesch, B. (1997). Prion (PrPSc)-specific epitope defined by a monoclonal antibody. *Nature,* **390,** 74-77.

Kosco, M. H., Pflugfelder, E. and Gray, D. (1992). Follicular dendritic cell-dependent adhesion and proliferation of B cells in vitro. *J Immunol,* **148,** 2331-2339.

Kosco-Vilbois, M. H. (2000). Follicular dendritic cells: a license to tangle with scrapie. *Immunol Today,* **21,** 468.

Kretzschmar, H. A., Tings, T., Madlung, A., Giese, A. and Herms, J. (2000). Function of PrP(C) as a copper-binding protein at the synapse. *Arch Virol Suppl,* **16,** 239-249.

Kucharzik, T., Lugering, N., Rautenberg, K., Lugering, A., Schmidt, M. A., Stoll, R. and Domschke, W. (2000). Role of M cells in intestinal barrier function. *Ann NY Acad Sci,* **915,** 171-183.

Landsverk, T. (1988). Phagocytosis and transcytosis by the follicle-associated epithelium of the ileal Peyer's patch in calves. *Immunol Cell Biol,* **66,** 261-268.

Landsverk, T., Halleraker, M., Aleksandersen, M., McClure, S., Hein, W. and Nicander, L. (1991). The intestinal habitat for organized lymphoid tissues in ruminants; comparative aspects of structure, function and development. *Vet Immunol and Immunopath,* **28,** 1-16.

Landsverk, T., Jansson, A., Nicander, L. and Ploen, L. (1987). Carbonic anhydrase--a marker for particles shed from the epithelium to the lymphoid follicles of the ileal Peyer's patch in goat kids and lambs. *Immunol Cell Biol,* **65,** 425-429.

Landsverk, T., Lium, B., Matovelo, J. A., Liven, E. and Nordstoga, K. (1990). Peyer's patches in experimental Salmonella dublin infection in calves. Microvascular and epithelial changes contributing to atrophy of lymphoid follicles. *Apmis,* **98,** 255-268.

Lantos, P. L., Bhatia, K., Doey, L. J., al-Sarraj, S., Doshi, R., Beck, J. and Collinge, J. (1997). Is the neuropathology of new variant Creutzfeldt-Jakob disease and kuru similar? *Lancet,* **350,** 187-188.

Lantos, P. L., McGill, I. S., Janota, I., Doey, L. J., Collinge, J., Bruce, M. T., Whatley, S. A., Anderton, B. H., Clinton, J., Roberts, G. W. and et al. (1992). Prion protein immunocytochemistry helps to establish the true incidence of prion diseases. *Neurosci Lett,* **147,** 67-71.

Laplanche, J. L., Chatelain, J., Westaway, D., Thomas, S., Dussaucy, M., Brugere-Picoux, J. and Launay, J. M. (1993). PrP polymorphisms associated with natural scrapie discovered by denaturing gradient gel electrophoresis. *Genomics,* **15,** 30-37.

Laplanche, J. L., Elsen, J.-M., Eychenne, F., Schelcher, F., Richard, S., Amigues, Y. and Launay, J. M. (1996). Scrapie outbreak in sheep after oral exposure to infective gastrointestinal nematode larvae. In: *Transmissible Subacute Spongiform Encephalopathies: Prion Diseases.*, L. Court and B. Dodet, Eds, Elsevier, Paris, pp. 41-46.

Laplanche, J. L., Hachimi, K. H., Durieux, I., Thuillet, P., Defebvre, L., Delasnerie-Laupretre, N., Peoc'h, K., Foncin, J. F. and Destee, A. (1999). Prominent psychiatric features and early onset in an inherited prion disease with a new insertional mutation in the prion protein gene. *Brain,* **122,** 2375-2386.

Larsen, H. J. and Landsverk, T. (1986). Distribution of T and B lymphocytes in jejunal and ileocaecal Peyer's patches of lambs. *Res Vet Sci,* **40,** 105-111.

Lasmézas, C. I. (2003). The transmissible spongiform encephalopathies. *Rev Sci Tech,* **22,** 23-36.

Lasmézas, C. I., Cesbron, J. Y., Deslys, J. P., Demaimay, R., Adjou, K. T., Rioux, R., Lemaire, C., Locht, C. and Dormont, D. (1996). Immune system-dependent and -independent replication of the scrapie agent. *J Virol,* **70,** 1292-1295.

Lasmézas, C. I., Comoy, E., Hawkins, S., Herzog, C., Mouthon, F., Konold, T., Auvre, F., Correia, E., Lescoutra-Etchegaray, N., Sales, N., Wells, G., Brown, P. and Deslys, J. P. (2005). Risk of oral infection with bovine spongiform encephalopathy agent in primates. *Lancet,* **365,** 781-783.

Lasmézas, C. I., Deslys, J. P., Robain, O., Jaegly, A., Beringue, V., Peyrin, J. M., Fournier, J. G., Hauw, J. J., Rossier, J. and Dormont, D. (1997). Transmission of the BSE agent to mice in the absence of detectable abnormal prion protein. *Science,* **275,** 402-405.

Le Dur, A., Beringue, V., Andreoletti, O., Reine, F., Lai, T. L., Baron, T., Bratberg, B., Vilotte, J. L., Sarradin, P., Benestad, S. L. and Laude, H. (2005). A newly identified type of scrapie agent can naturally infect sheep with resistant PrP genotypes. *Proc Natl Acad Sci U S A,* **102,** 16031-16036.

Le Hir, M., Bluethmann, H., Kosco-Vilbois, M. H., Muller, M., di Padova, F., Moore, M., Ryffel, B. and Eugster, H. P. (1996). Differentiation of follicular dendritic cells and full antibody responses require tumor necrosis factor receptor-1 signaling. *J Exp Med,* **183,** 2367-2372.

Lee, H. S., Brown, P., Cervenakova, L., Garruto, R. M., Alpers, M. P., Gajdusek, D. C. and Goldfarb, L. G. (2001). Increased susceptibility to Kuru of carriers of the PRNP 129 methionine/methionine genotype. *J Infect Dis,* **183,** 192-196.

Lichtman, J. (1977). The reorganisation of synaptic connections in the rat submandibular ganglion during post-natal development. *J Physiol,* **273,** 155-177.

Lie, K. I., Aleksandersen, M. and Landsverk, T. (2005). Lymphoid follicles of different phenotype appear in ileum during involution of the sheep ileal Peyer's patch. *Dev Comp Immunol,* **29,** 539-553.

Liebler, E. M., Pohlenz, J. F. and Woode, G. N. (1988). Gut-associated lymphoid tissue in the large intestine of calves. I. Distribution and histology. *Vet Pathol,* **25,** 503-508.

Liu, T., Li, R., Wong, B. S., Liu, D., Pan, T., Petersen, R. B., Gambetti, P. and Sy, M. S. (2001). Normal cellular prion protein is preferentially expressed on subpopulations of murine hemopoietic cells. *J Immunol,* **166,** 3733-3742.

Liu, Y. J., Xu, J., de Bouteiller, O., Parham, C. L., Grouard, G., Djossou, O., de Saint-Vis, B., Lebecque, S., Banchereau, J. and Moore, K. W. (1997). Follicular dendritic cells specifically express the long CR2/CD21 isoform. *J Exp Med,* **185,** 165-170.

Lupi, O. (2003). Could ectoparasites act as vectors for prion diseases? *Int J Dermatol,* **42,** 425-429.

Ma, J. and Lindquist, S. (2002). Conversion of PrP to a Self-Perpetuating PrP^{Sc}-like conformation in the cytosol. *Science,* **298,** 1785-1788.

Ma, J., Wollmann, R. and Lindquist, S. (2002). Neurotoxicity and neurodegeneration when PrP accumulates in the cytosol. *Science,* **298,** 1781-1785.

Mabbott, N. A., Brown, K. L., Manson, J. and Bruce, M. E. (1997). T-lymphocyte activation and the cellular form of the prion protein. *Immunology,* **92,** 161-165.

Mabbott, N. A. and Bruce, M. E. (2001). The immunobiology of TSE diseases. *J Gen Virol,* **82,** 2307-2318.

Mabbott, N. A., Farquhar, C. F., Brown, K. L. and Bruce, M. E. (1998). Involvement of the immune system in TSE pathogenesis. *Immunol Today,* **19,** 201-203.

Mabbott, N. A., Mackay, F., Minns, F. and Bruce, M. E. (2000). Temporary inactivation of follicular dendritic cells delays neuroinvasion of scrapie. *Nat Med,* **6,** 719-720.

Mabbott, N. A., Young, J., McConnell, I. and Bruce, M. E. (2003). Follicular dendritic cell dedifferentiation by treatment with an inhibitor of the lymphotoxin pathway dramatically reduces scrapie susceptibility. *J Virol,* **77,** 6845-6854.

MacDonald, T. T. and Spencer, J. (1994). Ontogeny of the gut-associated lymphoid system in man. *Acta Paediatr Suppl,* **83,** 3-5.

Mackay, F. and Browning, J. L. (1998). Turning off follicular dendritic cells. *Nature,* **395,** 26-27.

MacLennan, I. C. (1994). Germinal centers. *Annu Rev Immunol,* **12,** 117-139.

Maignien, T., Lasmézas, C. I., Beringue, V., Dormont, D. and Deslys, J. P. (1999). Pathogenesis of the oral route of infection of mice with scrapie and bovine spongiform encephalopathy agents. *J Gen Virol,* **80,** 3035-3042.

Manolakou, K., Beaton, J., McConnell, I., Farquar, C., Manson, J., Hastie, N. D., Bruce, M. and Jackson, I. J. (2001). Genetic and environmental factors modify bovine spongiform encephalopathy incubation period in mice. *Proc Natl Acad Sci U S A,* **98,** 7402-7407.

Manson, J. C., Jamieson, E., Baybutt, H., Tuzi, N. L., Barron, R., McConnell, I., Somerville, R., Ironside, J., Will, R., Sy, M. S., Melton, D. W., Hope, J. and Bostock, C. (1999). A single amino acid alteration (101L) introduced into murine PrP dramatically alters incubation time of transmissible spongiform encephalopathy. *Embo J,* **18,** 6855-6864.

Marsh, R. F. and Bessen, R. A. (1993). Epidemiologic and experimental studies on transmissible mink encephalopathy. *Dev Biol Stand,* **80,** 111-118.

Marsh, R. F. and Hadlow, W. J. (1992). Transmissible mink encephalopathy. *Rev Sci Tech,* **11,** 539-550.

Marshall-Clarke, S., Tasker, L. and Parkhouse, R. M. (2000). Immature B lymphocytes from adult bone marrow exhibit a selective defect in induced hyperexpression of major histocompatibility complex class II and fail to show B7.2 induction. *Immunology,* **100,** 141-151.

Masters, C. L., Harris, J. O., Gajdusek, D. C., Gibbs, C. J., Jr., Bernoulli, C. and Asher, D. M. (1979). Creutzfeldt-Jakob disease: patterns of worldwide occurrence and the significance of familial and sporadic clustering. *Ann Neurol,* **5,** 177-188.

Matthews, L., Coen, P. G., Foster, J. D., Hunter, N. and Woolhouse, M. E. (2001). Population dynamics of a scrapie outbreak. *Arch Virol,* **146,** 1173-1186.

Mayrhofer, G. (1984). Physiology of the intestinal immune response. In: *Local Immune Responses of the Gut*, T. Newby and C. Stokes, Eds, CRC Press, Inc, Florida, United States, pp. 1-96.

McBride, P. A., Eikelenboom, P., Kraal, G., Fraser, H. and Bruce, M. E. (1992). PrP protein is associated with follicular dendritic cells of spleens and lymph nodes in uninfected and scrapie-infected mice. *J Pathol,* **168,** 413-418.

McFarlin, D. E., Raff, M. C., Simpson, E. and Nehlsen, S. H. (1971). Scrapie in immunologically deficient mice. *Nature,* **233,** 336.

McInyyre, K.M., Gubbins, S., Goldmann, W., Stevenson, E. and Baylis, M. The time-course of a scrapie outbreak. *BMC Veterinary Reseacrh,* **2,** 20.

McLean, A. R. and Bostock, C. J. (2000). Scrapie infections initiated at varying doses: an analysis of 117 titration experiments. *Philos Trans R Soc Lond B Biol Sci,* **355,** 1043-1050.

Merz, P. A., Rohwer, R. G., Kascsak, R., Wisniewski, H. M., Somerville, R. A., Gibbs, C. J., Jr. and Gajdusek, D. C. (1984). Infection-specific particle from the unconventional slow virus diseases. *Science,* **225,** 437-440.

Merz, P. A., Somerville, R. A., Wisniewski, H. M. and Iqbal, K. (1981). Abnormal fibrils from scrapie-infected brain. *Acta Neuropathol (Berl),* **54,** 63-74.

Meyer, N., Rosenbaum, V., Schmidt, B., Gilles, K., Mirenda, C., Groth, D., Prusiner, S. B. and Riesner, D. (1991). Search for a putative scrapie genome in purified prion fractions reveals a paucity of nucleic acids. *J Gen Virol,* **72,** 37-49.

Miller, M. W. and Williams, E. S. (2003). Prion disease: horizontal prion transmission in mule deer. *Nature,* **425,** 35-36.

Miller, M. W., Williams, E. S., McCarty, C. W., Spraker, T. R., Kreeger, T. J., Larsen, C. T. and Thorne, E. T. (2000). Epizootiology of chronic wasting disease in free-ranging cervids in Colorado and Wyoming. *J Wildl Dis,* **36,** 676-690.

Millson, G. C. and Manning, E. J. (1979). *Slow Transmissible Diseases of the Nervous System.* Academic Press, New York, pp. 409-424.

Mirsky, R. and Jessen, K. R. (1999). The neurobiology of Schwann cells. *Brain Pathol,* **9,** 293-311.

Moghaddami, M., Cummins, A. and Mayrhofer, G. (1998). Lymphocyte-filled villi: comparison with other lymphoid aggregations in the mucosa of the human small intestine. *Gastroenterology,* **115,** 1414-1425.

Mohan, J, Brown, K.L., Farquhar, C.F., Bruce, M.E. and Mabbott, N.A. (2004). Scrapie transmission following exposure through the skin is dependent on follicular dendritic cells in lymphoid tissues. *Journal of Dermatological Science,* **35,** 101-111.

Momotani, E., Whipple, D. L., Thiermann, A. B. and Cheville, N. F. (1988). Role of M cells and macrophages in the entrance of Mycobacterium paratuberculosis into domes of ileal Peyer's patches in calves. *Vet Pathol,* **25,** 131-137.

Monleon, E., Monzon, M., Hortells, P., Vargas, A., Acin, C. and Badiola, J. J. (2004). Detection of PrPsc on lymphoid tissues from naturally affected scrapie animals: comparison of three visualization systems. *J Histochem Cytochem,* **52,** 145-151.

Montrasio, F., Frigg, R., Glatzel, M., Klein, M. A., Mackay, F., Aguzzi, A. and Weissmann, C. (2000). Impaired prion replication in spleens of mice lacking functional follicular dendritic cells. *Science,* **288,** 1257-1259.

Moolenbeek, C. and Ruitenberg, E. J. (1981). The "Swiss roll": a simple technique for histological studies of the rodent intestine. *Lab Anim,* **15,** 57-59.

Moore, R. C., Hope, J., McBride, P. A., McConnell, I., Selfridge, J., Melton, D. W. and Manson, J. C. (1998). Mice with gene targetted prion protein alterations show that Prnp, Sinc and Prni are congruent. *Nat Genet,* **18,** 118-125.

Moore, R. C., Lee, I. Y., Silverman, G. L., Harrison, P. M., Strome, R., Heinrich, C., Karunaratne, A., Pasternak, S. H., Chishti, M. A., Liang, Y., Mastrangelo, P., Wang, K., Smit, A. F., Katamine, S., Carlson, G. A., Cohen, F. E., Prusiner, S. B., Melton, D. W., Tremblay, P., Hood, L. E. and Westaway, D. (1999). Ataxia in prion protein (PrP)-deficient mice is associated with upregulation of the novel PrP-like protein doppel. *J Mol Biol,* **292,** 797-817.

Moreno, C., Lantier, I. and Lantier, F. (2002). Transposition to sheep of mouse quantitative trait loci (QTL) influencing susceptibility to prion diseases. *Proceedings of the Seventh World Congress on Genetics Applied to Livestock Production*, Montpellier, France, 19-23 August 2002, pp. 13-21.

Moreno, C. R., Lantier, F., Lantier, I., Sarradin, P. and Elsen, J. M. (2003). Detection of new quantitative trait Loci for susceptibility to transmissible spongiform encephalopathies in mice. *Genetics,* **165,** 2085-2091.

Mouillet-Richard, S., Ermonval, M., Chebassier, C., Laplanche, J. L., Lehmann, S., Launay, J. M. and Kellermann, O. (2000). Signal transduction through prion protein. *Science,* **289,** 1925-1928.

Neibergs, H. L., Ryan, A. M., Womack, J. E., Spooner, R. L. and Williams, J. L. (1994). Polymorphism analysis of the prion gene in BSE-affected and unaffected cattle. *Anim Genet,* **25,** 313-317.

Nicoletti, C. (2000). Unsolved mysteries of intestinal M cells. *Gut,* **47,** 735-739.

Oesch, B., Westaway, D., Walchli, M., McKinley, M. P., Kent, S. B., Aebersold, R., Barry, R. A., Tempst, P., Teplow, D. B., Hood, L. E. and et al. (1985). A cellular gene encodes scrapie PrP 27-30 protein. *Cell,* **40,** 735-746.

O'Rourke, K. I., Baszler, T. V., Miller, J. M., Spraker, T. R., Sadler-Riggleman, I. and Knowles, D. P. (1998). Monoclonal antibody F89/160.1.5 defines a conserved epitope on the ruminant prion protein. *J Clin Microbiol,* **36,** 1750-1755.

O'Rourke, K. I., Besser, T. E., Miller, M. W., Cline, T. F., Spraker, T. R., Jenny, A. L., Wild, M. A., Zebarth, G. L. and Williams, E. S. (1999). PrP genotypes of captive and free-ranging Rocky Mountain elk (Cervus elaphus nelsoni) with chronic wasting disease. *J Gen Virol,* **80,** 2765-2769.

O'Rourke, K. I., Holyoak, G. R., Clark, W. W., Mickelson, J. R., Wang, S., Melco, R. P., Besser, T. E. and Foote, W. C. (1997). PrP genotypes and experimental scrapie in orally inoculated Suffolk sheep in the United States. *J Gen Virol,* **78,** 975-978.

Outram, G. W. (1976). The pathogenesis of scrapie in mice. In: *Slow Virus Diseases of Animals and Man*, R. Kimberlain, Ed, American Elsevier Publishing Company, INC., New York, USA.

Outram, G. W., Dickinson, A. G. and Fraser, H. (1973). Developmental maturation of susceptibility to scrapie in mice. *Nature,* **241,** 536-537.

Pabst, R., Geist, M., Rothkotter, H. J. and Fritz, F. J. (1988). Postnatal development and lymphocyte production of jejunal and ileal Peyer's patches in normal and gnotobiotic pigs. *Immunology,* **64,** 539-544.

Paitel, E., Alves da Costa, C., Vilette, D., Grassi, J. and Checler, F. (2002). Overexpression of PrPc triggers caspase 3 activation: potentiation by proteasome inhibitors and blockade by anti-PrP antibodies. *J Neurochem,* **83,** 1208-1214.

Pammer, J., Cross, H. S., Frobert, Y., Tschachler, E. and Oberhuber, G. (2000). The pattern of prion-related protein expression in the gastrointestinal tract. *Virchows Arch,* **436,** 466-472.

Pammer, J., Weninger, W. and Tschachler, E. (1998). Human keratinocytes express cellular prion-related protein in vitro and during inflammatory skin diseases. *Am J Pathol,* **153,** 1353-1358.

Pan, K. M., Baldwin, M., Nguyen, J., Gasset, M., Serban, A., Groth, D., Mehlhorn, I., Huang, Z., Fletterick, R. J., Cohen, F. E. and et al. (1993). Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion proteins. *Proc Natl Acad Sci U S A,* **90,** 10962-10966.

Parry, H. (1983). *Scrapie disease in sheep*. Academic Press, London.

Parry, H. B. (1962). Scrapie: a transmissible and hereditary disease of sheep. *Heredity,* **17,** 75-105.

Partanen, J. (2003). Genetic susceptibility to variant Creutzfeldt-Jakob disease. *Lancet,* **361,** 447-448.

Pasparakis, M., Alexopoulou, L., Episkopou, V. and Kollias, G. (1996). Immune and inflammatory responses in TNF alpha-deficient mice: a critical requirement for TNF alpha in the formation of primary B cell follicles, follicular dendritic cell networks and germinal centers, and in the maturation of the humoral immune response. *J Exp Med,* **184,** 1397-1411.

Pattison, I. H., Hoare, M. N., Jebbett, J. N. and Watson, W. A. (1972). Spread of scrapie to sheep and goats by oral dosing with foetal membranes from scrapie-affected sheep. *Vet Rec,* **90,** 465-468.

Peden, A. H. and Ironside, J. W. (2004). Review: pathology of variant Creutzfeldt-Jakob disease. *Folia Neuropathol,* **42 Suppl A,** 85-91.

Petrie, A. and Watson, P. (2001). *Statistics for Veterinary and Animal Science*. Blackwell Science Ltd., London.

Poskitt, D. C., Duffey, K., Barnett, J., Kimpton, W. G. and Muller, H. K. (1984). The gut-associated lymphoid system of two species of Australian marsupial mice, Antechinus swainsonii and Antechinus stuartii. Distribution, frequency and structure of Peyer's patches and lymphoid follicles in the small and large intestine. *Aust J Exp Biol Med Sci,* **62,** 81-88.

Press, C. M., Heggebø, R. and Espenes, A. (2004). Involvement of gut-associated lymphoid tissue of ruminants in the spread of transmissible spongiform encephalopathies. *Adv Drug Deliv Rev,* **56,** 885-899.

Prinz, M., Huber, G., Macpherson, A. J., Heppner, F. L., Glatzel, M., Eugster, H. P., Wagner, N. and Aguzzi, A. (2003). Oral prion infection requires normal numbers of Peyer's patches but not of enteric lymphocytes. *Am J Pathol,* **162,** 1103-1011.

Prusiner, S. B. (1982). Novel proteinaceous infectious particles cause scrapie. *Science,* **216,** 136-144.

Prusiner, S. B. (1991). Molecular biology of prion diseases. *Science,* **252,** 1515-1522.

Prusiner, S. B., Cochran, S. P. and Alpers, M. P. (1985). Transmission of scrapie in hamsters. *J Infect Dis,* **152,** 971-978.

Prusiner, S. B., Cochran, S. P., Downey, D. E. and Groth, D. F. (1981). Determination of scrapie agent titer from incubation period measurements in hamsters. *Adv Exp Med Biol,* **134,** 385-399.

Prusiner, S. B., Groth, D. F., Bolton, D. C., Kent, S. B. and Hood, L. E. (1984). Purification and structural studies of a major scrapie prion protein. *Cell,* **38,** 127-134.

Prusiner, S. B., Scott, M., Foster, D., Pan, K. M., Groth, D., Mirenda, C., Torchia, M., Yang, S. L., Serban, D., Carlson, G. A. and et al. (1990). Transgenetic studies implicate interactions between homologous PrP isoforms in scrapie prion replication. *Cell,* **63,** 673-686.

Prusiner, S. B., Scott, M. R., DeArmond, S. J. and Cohen, F. E. (1998). Prion protein biology. *Cell,* **93,** 337-348.

Radoux, D., Heinen, E., Kinet-Denoel, C., Tihange, E. and Simar, L. (1984). Precise localization of antigens on follicular dendritic cells. *Cell Tissue Res,* **235,** 267-274.

Raeber, A. J., Klein, M. A., Frigg, R., Flechsig, E., Aguzzi, A. and Weissmann, C. (1999). PrP-dependent association of prions with splenic but not circulating lymphocytes of scrapie-infected mice. *Embo J,* **18,** 2702-2706.

Redman, C., Coen, P., Matthews, L., Lewis, R., Dingwall, W., Foster, J., Chase-Topping, M., Hunter, N. and Woolhouse, M. (2002). Comparative epidemiology of scrapie outbreaks in individual sheep flocks. *Epidemiol Infect,* **128,** 513-521.

Reilly, R. W. and Kirsner, J. B. (1965). Runt Intestinal Disease. *Lab Invest,* **14,** 102-107.

Reynaud, C. A., Garcia, C., Hein, W. R. and Weill, J. C. (1995). Hypermutation generating the sheep immunoglobulin repertoire is an antigen-independent process. *Cell,* **80,** 115-125.

Reynaud, C. A., Mackay, C. R., Muller, R. G. and Weill, J. C. (1991). Somatic generation of diversity in a mammalian primary lymphoid organ: the sheep ileal Peyer's patches. *Cell,* **64,** 995-1005.

Reynolds, J. (1980). Gut-associated lymphoid tissues in lambs before and after birth. *Monogr Allergy,* **16,** 187-202.

Reynolds, J., Pabst, R. and Bordmann, G. (1985). Evidence for the existence of two distinct types of Peyer's patches in sheep. *Adv Exp Med Biol,* **186,** 101-109.

Reynolds, J. D. (1987). Peyer's patches and the early development of B lymphocytes. *Curr Top Microbiol Immunol,* **135,** 43-56.

Reynolds, J. D. and Morris, B. (1983). The evolution and involution of Peyer's patches in fetal and postnatal sheep. *Eur J Immunol,* **13,** 627-635.

Reynolds, J. D. and Pabst, R. (1984). The emigration of lymphocytes from Peyer's patches in sheep. *Eur J Immunol,* **14,** 7-13.

Rieger, R., Edenhofer, F., Lasmézas, C. I. and Weiss, S. (1997). The human 37-kDa laminin receptor precursor interacts with the prion protein in eukaryotic cells. *Nat Med,* **3,** 1383-1388.

Riek, R., Hornemann, S., Wider, G., Glockshuber, R. and Wuthrich, K. (1997). NMR characterization of the full-length recombinant murine prion protein, mPrP(23-231). *FEBS Lett,* **413,** 282-288.

Ritchie, D., Brown, K. and Bruce, M. (1999). Visualisation of PrP protein and follicular dendritic cells in uninfected and scrapie infected spleen. *J Cell Path,* **1,** 3-10.

Robakis, N. K., Sawh, P. R., Wolfe, G. C., Rubenstein, R., Carp, R. I. and Innis, M. A. (1986). Isolation of a cDNA clone encoding the leader peptide of prion protein and expression of the homologous gene in various tissues. *Proc Natl Acad Sci U S A,* **83,** 6377-6381.

Roesler, R., Walz, R., Quevedo, J., de-Paris, F., Zanata, S. M., Graner, E., Izquierdo, I., Martins, V. R. and Brentani, R. R. (1999). Normal inhibitory avoidance learning and anxiety, but increased locomotor activity in mice devoid of PrP(C). *Brain Res Mol Brain Res,* **71,** 349-353.

Ronne, M. (1989). Chromosome preparation and high resolution banding techniques. A review. *J Dairy Sci,* **72,** 1363-1377.

Ryder, S. J., Wells, G. A., Bradshaw, J. M. and Pearson, G. R. (2001). Inconsistent detection of PrP in extraneural tissues of cats with feline spongiform encephalopathy. *Vet Rec,* **148,** 437-441.

Safar, J., Wille, H., Itri, V., Groth, D., Serban, H., Torchia, M., Cohen, F. E. and Prusiner, S. B. (1998). Eight prion strains have PrP(Sc) molecules with different conformations. *Nat Med,* **4,** 1157-1165.

Sailer, A., Büeler, H., Fischer, M., Aguzzi, A. and Weissmann, C. (1994). No propagation of prions in mice devoid of PrP. *Cell,* **77,** 967-968.

Sakaguchi, S., Katamine, S., Nishida, N., Moriuchi, R., Shigematsu, K., Sugimoto, T., Nakatani, A., Kataoka, Y., Houtani, T., Shirabe, S., Okada, H., Hasegawa, S., Miyamoto, T. and Noda, T. (1996). Loss of cerebellar Purkinje cells in aged mice homozygous for a disrupted PrP gene. *Nature,* **380,** 528-531.

Sales, N., Hassig, R., Rodolfo, K., Di Giamberardino, L., Traiffort, E., Ruat, M., Fretier, P. and Moya, K. L. (2002). Developmental expression of the cellular prion protein in elongating axons. *Eur J Neurosci,* **15,** 1163-1177.

Sander, P., Hamann, H., Pfeiffer, I., Wemheuer, W., Brenig, B., Groschup, M. H., Ziegler, U., Distl, O. and Leeb, T. (2004). Analysis of sequence variability of the bovine prion protein gene (PRNP) in German cattle breeds. *Neurogenetics,* **5,** 19-25.

Schreuder, B. E., van Keulen, L. J., Vromans, M. E., Langeveld, J. P. and Smits, M. A. (1998). Tonsillar biopsy and PrPSc detection in the preclinical diagnosis of scrapie. *Vet Rec,* **142,** 564-568.

Schriever, F., Freedman, A. S., Freeman, G., Messner, E., Lee, G., Daley, J. and Nadler, L. M. (1989). Isolated human follicular dendritic cells display a unique antigenic phenotype. *J Exp Med,* **169,** 2043-2058.

Schuler, G., Thurner, B. and Romani, N. (1997). Dendritic cells: from ignored cells to major players in T-cell-mediated immunity. *Int Arch Allergy Immunol,* **112,** 317-322.

Schulz-Schaeffer, W. J., Fatzer, R., Vandevelde, M. and Kretzschmar, H. A. (2000a). Detection of PrP(Sc) in subclinical BSE with the paraffin-embedded tissue (PET) blot. *Arch Virol Suppl,* **16,** 173-180.

Schulz-Schaeffer, W. J., Tschoke, S., Kranefuss, N., Drose, W., Hause-Reitner, D., Giese, A., Groschup, M. H. and Kretzschmar, H. A. (2000b). The paraffin-embedded tissue blot detects PrP(Sc) early in the incubation time in prion diseases. *Am J Pathol,* **156,** 51-56.

Scott, M., Foster, D., Mirenda, C., Serban, D., Coufal, F., Walchli, M., Torchia, M., Groth, D., Carlson, G., DeArmond, S. J. and et al. (1989). Transgenic mice expressing hamster prion protein produce species-specific scrapie infectivity and amyloid plaques. *Cell,* **59,** 847-857.

Sellheyer, K., Schwarting, R. and Stein, H. (1989). Isolation and antigenic profile of follicular dendritic cells. *Clin Exp Immunol,* **78,** 431-436.

Shlomchik, M. J., Radebold, K., Duclos, N. and Manuelidis, L. (2001). Neuroinvasion by a Creutzfeldt-Jakob disease agent in the absence of B cells and follicular dendritic cells. *Proc Natl Acad Sci U S A,* **98,** 9289-9294.

Shmakov, A. and Ghosh, S. (2001). Prion proteins and the gut: une liaison dangereuse? *Gut,* **48,** 443-447.

Shmakov, A. N., Bode, J., Kilshaw, P. J. and Ghosh, S. (2000). Diverse patterns of expression of the 67-kD laminin receptor in human small intestinal mucosa: potential binding sites for prion proteins? *J Pathol,* **191,** 318-322.

Shmerling, D., Hegyi, I., Fischer, M., Blättler, T., Brandner, S., Gotz, J., Rulicke, T., Flechsig, E., Cozzio, A., von Mering, C., Hangartner, C., Aguzzi, A. and Weissmann, C. (1998). Expression of amino-terminally truncated PrP in the mouse leading to ataxia and specific cerebellar lesions. *Cell,* **93,** 203-214.

Shortman, K., Wu, L., Suss, G., Kronin, V., Winkel, K., Saunders, D. and Vremec, D. (1997). Dendritic cells and T lymphocytes: developmental and functional interactions. *Ciba Found Symp,* **204,** 130-141.

Sigurdarson, S. (1991). Epidemiology of scrapie in Iceland and experience with control measures. In: *Sub-acute Spongiform Encephalopathies*, R. Bradley, M. Savey and B. Marchant, Eds, Kluwer Academic Publishers, Dordrecht, pp. 233-242.

Sigurdson, C. J., Barillas-Mury, C., Miller, M. W., Oesch, B., van Keulen, L. J., Langeveld, J. P. and Hoover, E. A. (2002). PrP(CWD) lymphoid cell targets in early and advanced chronic wasting disease of mule deer. *J Gen Virol,* **83,** 2617-2628.

Sigurdson, C. J., Williams, E. S., Miller, M. W., Spraker, T. R., O'Rourke, K. I. and Hoover, E. A. (1999). Oral transmission and early lymphoid tropism of chronic wasting disease PrP^{res} in mule deer fawns (*Odocoileus hemionus*). *J Gen Virol,* **80,** 2757-2764.

Sigurdsson, B. (1954). Rida, a chronic encephalitis of sheep with general remarks on infections which develop slowly and some of their special characteristics. *British Veterinary Journal,* 341-354.

Sminia, T., Wilders, M. M. and Janse. (1983). Antigen-processing cells in gut associated lymphoid tissue (GALT). *Cell Biol Int Rep,* **7,** 677.

Smith, P. G. and Bradley, R. (2003). Bovine spongiform encephalopathy (BSE) and its epidemiology. *Br Med Bull,* **66,** 185-198.

Somerville, R. A. (1985). Ultrastructural links between scrapie and Alzheimer's disease. *Lancet,* **1,** 504-506.

Somerville, R. A., Birkett, C. R., Farquhar, C. F., Hunter, N., Goldmann, W., Dornan, J., Grover, D., Hennion, R. M., Percy, C., Foster, J. and Jeffrey, M. (1997). Immunodetection of PrPSc in spleens of some scrapie-infected sheep but not BSE-infected cows. *J Gen Virol,* **78,** 2389-2396.

Sparkes, R. S., Simon, M., Cohn, V. H., Fournier, R. E., Lem, J., Klisak, I., Heinzmann, C., Blatt, C., Lucero, M., Mohandas, T. and et al. (1986). Assignment of the human and mouse prion protein genes to homologous chromosomes. *Proc Natl Acad Sci U S A,* **83,** 7358-7362.

Stahl, N. and Prusiner, S. B. (1991). Prions and prion proteins. *Faseb J,* **5,** 2799-2807.

Steinman, R. M. (1996). Dendritic cells and immune-based therapies. *Exp Hematol,* **24,** 859-862.

Szakal, A. K., Kapasi, Z. F., Masuda, A. and Tew, J. G. (1992). Follicular dendritic cells in the alternative antigen transport pathway: microenvironment, cellular events, age and retrovirus related alterations. *Semin Immunol,* **4,** 257-265.

Sy, M. S. and Gambetti, P. (1999). Prion replication-once again blaming the dendritic cell. *Nat Med,* **5,** 1235-1237.

Taylor, D. M., McConnell, I. and Fraser, H. (1996). Scrapie infection can be established readily through skin scarification in immunocompetent but not immunodeficient mice. *J Gen Virol,* **77,** 1595-1599.

Taylor, P. R., Pickering, M. C., Kosco-Vilbois, M. H., Walport, M. J., Botto, M., Gordon, S. and Martinez-Pomares, L. (2002). The follicular dendritic cell restricted epitope, FDC-M2, is complement C4; localization of immune complexes in mouse tissues. *Eur J Immunol,* **32,** 1888-1896.

Telling, G. C., Scott, M., Mastrianni, J., Gabizon, R., Torchia, M., Cohen, F. E., DeArmond, S. J. and Prusiner, S. B. (1995). Prion propagation in mice expressing human and chimeric PrP transgenes implicates the interaction of cellular PrP with another protein. *Cell,* **83,** 79-90.

Terry, L. A., Marsh, S., Ryder, S. J., Hawkins, S. A., Wells, G. A. and Spencer, Y. I. (2003). Detection of disease-specific PrP in the distal ileum of cattle exposed orally to the agent of bovine spongiform encephalopathy. *Vet Rec,* **152,** 387-392.

Tew, J. G., Kosco, M. H., Burton, G. F. and Szakal, A. K. (1990). Follicular dendritic cells as accessory cells. *Immunol Rev,* **117,** 185-211.

Tew, J. G., Thorbecke, G. J. and Steinman, R. M. (1982). Dendritic cells in the immune response: characteristics and recommended nomenclature (A report from the Reticuloendothelial Society Committee on Nomenclature). *J Reticuloendothel Soc,* **31,** 371-380.

Tew, J. G., Wu, J., Qin, D., Helm, S., Burton, G. F. and Szakal, A. K. (1997). Follicular dendritic cells and presentation of antigen and costimulatory signals to B cells. *Immunol Rev,* **156,** 39-52.

Thackray, A. M., Klein, M. A., Aguzzi, A. and Bujdoso, R. (2002). Chronic subclinical prion disease induced by low-dose inoculum. *J Virol,* **76,** 2510-2517.

Therneau, T. M. and Grambsch, P. M. (2000). *Modeling Survival Data: Extending the Cox Model.* Springer, New York.

Thielen, C., Melot, F., Jolois, O., Leclercq, F., Tsunoda, R., Frobert, Y., Heinen, E. and Antoine, N. (2001). Isolation of bovine follicular dendritic cells allows the demonstration of a particular cellular prion protein. *Cell Tissue Res,* **306,** 49-55.

Timens, W., Rozeboom, T. and Poppema, S. (1987). Fetal and neonatal development of human spleen: an immunohistological study. *Immunology,* **60,** 603-609.

Tkachuk, M., Bolliger, S., Ryffel, B., Pluschke, G., Banks, T. A., Herren, S., Gisler, R. H. and Kosco-Vilbois, M. H. (1998). Crucial role of tumor necrosis factor receptor 1 expression on nonhematopoietic cells for B cell localization within the splenic white pulp. *J Exp Med,* **187,** 469-477.

Tobler, I., Gaus, S. E., Deboer, T., Achermann, P., Fischer, M., Rulicke, T., Moser, M., Oesch, B., McBride, P. A. and Manson, J. C. (1996). Altered circadian activity rhythms and sleep in mice devoid of prion protein. *Nature,* **380,** 639-642.

Tyler, K. L. (2004). COMMENTARY: Gibbs CJ Jr, Amyx HL, Bacote A, Masters CL, Gajdusek DC. Oral transmission of kuru, Creutzfeldt-Jakob disease, and scrapie to nonhuman primates. J Infect Dis 1980; 142:205-208. *J Infect Dis,* **190,** 653-660.

Valleron, A. J., Boëlle, P. Y., Will, R. and Cesbron, J. Y. (2001). Estimation of epidemic size and incubation time based on age characteristics of vCJD in the United Kingdom. *Science,* **294,** 1726-1728.

van den Berg, T. and Dijkistra, C. (1995). Ontogenesis and phylogenesis of FDC. In: *Follicular Dendritic Cells in Normal and Pathological Conditions*, E. Heinen, Ed, Chapman and Hall, pp. 35-51.

van Keulen, L. J., Schreuder, B. E., Meloen, R. H., Mooij-Harkes, G., Vromans, M. E. and Langeveld, J. P. (1996). Immunohistochemical detection of prion protein in lymphoid tissues of sheep with natural scrapie. *J Clin Microbiol,* **34,** 1228-1231.

van Keulen, L. J., Schreuder, B. E., Vromans, M. E., Langeveld, J. P. and Smits, M. A. (1999). Scrapie-associated prion protein in the gastrointestinal tract of sheep with natural scrapie. *J Comp Pathol,* **121,** 55-63.

Van Keulen, L. J. M., Schreuder, B. E. C., Vromans, M. E. W., Langeveld, J. P. M. and Smits, M. A. (2000). Prion Diseases, Diagnosis and Pathogenesis. In: *Pathogenesis of Natural Scrapie in Sheep*, M. H. Groschup and H. A. Kretzschmar, Eds, Springer-Verlag, Vienna, pp. 57-71.

van Kruiningen, H. J., West, A. B., Freda, B. J. and Holmes, K. A. (2002). Distribution of Peyer's patches in the distal ileum. *Inflamm Bowel Dis,* **8,** 180-185.

van Rooijen, N. and Streefkerk, J. G. (1976). Autoradiography and immunohistoperoxidase techniques applied to the same tissue section. *J Immunol Methods,* **10,** 379-383.

Waksman, B. H. (1973). The homing pattern of thymus-derived lymphocytes in calf and neonatal mouse Peyer's patches. *J Immunol,* **111,** 878-884.

Weissmann, C. (1991). A 'unified theory' of prion propagation. *Nature,* **352,** 679-683.

Wells, G., Hawkins, S., Green, R., Austin, A., Dexter, I., Spencer, Y., Chaplin, M., Stack, M. and Dawson, M. (1998). Preliminary observations on the pathogenesis of experimental bovine spongiform encephalopathy (BSE): an update. *Vet Rec,* **142,** 103-106.

Wells, G. A., Dawson, M., Hawkins, S. A., Green, R. B., Dexter, I., Francis, M. E., Simmons, M. M., Austin, A. R. and Horigan, M. W. (1994). Infectivity in the ileum of cattle challenged orally with bovine spongiform encephalopathy. *Vet Rec,* **135,** 40-41.

Wells, G. A., Spiropoulos, J., Hawkins, S. A. and Ryder, S. J. (2005). Pathogenesis of experimental bovine spongiform encephalopathy: preclinical infectivity in tonsil and observations on the distribution of lingual tonsil in slaughtered cattle. *Vet Rec,* **156,** 401-407.

WHO (1999). WHO consultation on diagnostic procedures for transmissible spongiform encephalopathies: Need for reference reagents and reference panels, World Health Organisation, Geneva, Switzerland, pp. 1-16.

Wilesmith, J. W., Ryan, J. B. and Atkinson, M. J. (1991). Bovine spongiform encephalopathy: epidemiological studies on the origin. *Vet Rec,* **128,** 199-203.

Wilesmith, J. W., Wells, G. A., Cranwell, M. P. and Ryan, J. B. (1988). Bovine spongiform encephalopathy: epidemiological studies. *Vet Rec,* **123,** 638-644.

Wilesmith, J. W., Wells, G. A. H., Ryan, J. B. N., Gavier-Widen, D. and Simmons, M. M. (1997). A cohort study to examine maternally-associated risk factors for BSE. *Vet Rec,* **141,** 239-243.

Will, R. G. (2003). Acquired prion disease: iatrogenic CJD, variant CJD, kuru. *Br Med Bull,* **66,** 255-265.

Will, R. G., Ironside, J. W., Zeidler, M., Cousens, S. N., Estibeiro, K., Alperovitch, A., Poser, S., Pocchiari, M., Hofman, A. and Smith, P. G. (1996). A new variant of Creutzfeldt-Jakob disease in the UK. *Lancet,* **347,** 921-925.

Will, R. G., Zeidler, M., Stewart, G. E., MacLeod, M. A., Ironside, J. W., Cousens, S. N., MacKenzie, J., Estibeiro, K., Green, A. J. and Knight, R. S. (2000). Diagnosis of new variant Creutzfeldt-Jakob disease. *Annals in Neurology,* **47,** 575-582.

Williams, E. and Miller, M. (2003). Transmissible spongiform encephalopathies in non-domestic animals: origin, transmission and risk factors. *Revue Scientifique et Technique de L'Office International des Epizooties,* **22,** 145-156.

Williams, E., Miller, M., Kreeger, T., Khan, R. and Thorne, E. (2002). Chronic wasting disease of deer and elk: a review with recommendations for management. *J Wildl Manage,* **66,** 551-563.

Williams, E. S. and Miller, M. W. (2002). Chronic wasting disease in deer and elk in North America. *Rev Sci Tech,* **21,** 305-316.

Williams, E. S. and Young, S. (1980). Chronic wasting disease of captive mule deer: a spongiform encephalopathy. *J Wildl Dis,* **16,** 89-98.

Willoughby, K., Kelly, D. F., Lyon, D. G. and Wells, G. A. (1992). Spongiform encephalopathy in a captive puma (Felis concolor). *Vet Rec,* **131,** 431-434.

Wilson, D. R., Anderson, R. D. and Smith, W. (1950). Studies in scrapie. *J Comp Pathol,* **60,** 267-282.

Wolf, J. L. and Bye, W. A. (1984). The membraneous epithelial (M) cell and the mucosal immune system. *Ann Rev Med,* **35,** 95-112.

Woolhouse, M. E., Taylor, P., Matanhire, D. & Chandiwana, S. K. (1991). Acquired immunity and epidemiology of Schistosoma haematobium. *Nature* **351**, 757-9.

Woolhouse, M. E., Stringer, S. M., Matthews, L., Hunter, N. and Anderson, R. M. (1998). Epidemiology and control of scrapie within a sheep flock. *Proc R Soc Lond B,* **265,** 1205-1210.

Woolhouse, M. E., Coen, P., Matthews, L., Foster, J. D., Elsen, J. M., Lewis, R. M., Haydon, D. T. and Hunter, N. (2001). A centuries-long epidemic of scrapie in British sheep? *Trends Microbiol,* **9,** 67-70.

Wyatt, J. M., Pearson, G. R., Smerdon, T. N., Gruffydd-Jones, T. J., Wells, G. A. and Wilesmith, J. W. (1991). Naturally occurring scrapie-like spongiform encephalopathy in five domestic cats. *Vet Rec,* **129,** 233-236.

Yeung, M. M., Melgar, S., Baranov, V., Oberg, A., Danielsson, A., Hammarstrom, S. and Hammarstrom, M. L. (2000). Characterisation of mucosal lymphoid aggregates in ulcerative colitis: immune cell phenotype and TcR-gammadelta expression. *Gut,* **47,** 215-227.

Zeidler, M., Stewart, G., Cousens, S. N., Estibeiro, K. and Will, R. G. (1997). Codon 129 genotype and new variant CJD. *Lancet,* **350,** 668.

Appendix A: Data used for quantification of lymphoid tissue in NPU Cheviot sheep

Table A-1: Description of variables for Table A-2

Variable	Description of variable
Sheep ID	sheep identification
Sex	Male or female
Seln/genotype*	Where genotype (**genotype**) information was not available, selection line (**Seln_line**) (positive or negative) is provided
DOB	Date of birth of animal
Cull Date	Day on which animal was culled
Days	Age of animal (in days) at death (**Cull Date – DOB**)
Ileal PP (m)	Length of ileal PP in metres. The length of the ileal PP was not recorded in animals over 12 months of age because of the patchy appearance of the structure in older sheep
LF1	First lymphoid follicle (LF) count (for repeatability study)
LF2	Second LF count (for repeatability study)
LF per cm^2	Average lymphoid follicle LF count per cm^2 of ileal PP tissue
Area 1	Area of ileal PP tissue calculated as a percentage of total ileal tissue (first count for repeatability study)
Area 2	Area of ileal PP tissue calculated as a percentage of total ileal tissue (second count for repeatability study)
% PP tissue	Average area of ileal PP tissue ([Area 1 + Area 2]/2) calculated as a percentage (%) of total ileal tissue

Table A-2 Lymphoid folicle counts and percentage of PP tissue for NPU Cheviot sheep

Sheep ID	Sex	Seln/ genotype	DOB	Cull Date	Days	Ileal PP (m)	LF1	LF2	LF per cm^2	Area 1	Area 2	% PP tissue
Lamb 1	M	-	23/03/2005	23/03/2005	0	0.91	4448	4508	187	59.44	58.99	59.2
Lamb 2	F	-	23/03/2005	23/03/2005	0	1.11	7080	7236	298	57.34	56.19	56.8
Lamb 3	F	-	23/03/2005	23/03/2005	0	0.98	7820	7860	327	48.35	47.54	47.9
Lamb 4	M	-	23/03/2005	23/03/2005	0	1.21	7084	7144	296	54.82	54.33	54.6
Lamb 5	F	-	29/03/2005	29/03/2005	0	1.12	8956	8796	370	60.52	61.18	60.9
Lamb 6	F	-	25/03/2005	29/03/2005	4	0.99	6295	6204	260	50.56	50.77	50.7
76x94	F	AHQ/ARR	01/05/2003	08/09/2003	130	1.23	4836	4836	202	59.30	58.50	58.9
76x93	M	AHQ/ARR	01/05/2003	08/10/2003	160	1.15	3834	3846	160	60.50	60.40	60.5
76x95	M	ARQ/ARR	01/05/2003	08/10/2003	160	1.25	4236	4218	176	58.00	57.70	57.9
79x42	F	ARQ/AHQ	01/04/2005	05/10/2005	187	0.96	4504	4310	184	53.71	51.25	52.5
79x43	F	ARQ/AHQ	01/04/2005	05/10/2005	187	1.27	3652	3786	155	49.99	50.10	50.0
79x60	F	ARQ/ARR	04/04/2005	05/10/2005	184	1.25	4204	4012	171	52.82	53.00	52.9
77x18	M	AHQ/AHQ	07/05/2003	04/12/2003	211	1.16	3084	2874	124	42.70	42.60	42.7
76x90	M	AHQ/AHQ	01/05/2003	04/12/2003	217	1.27	3624	3582	150	48.80	48.90	48.9
77x06	M	AHQ/ARR	01/05/2003	04/12/2003	217	1.27	2712	2658	112	46.80	46.60	46.7
77x11	M	VRQ/VRQ	01/05/2003	04/12/2003	217	1.08	3120	2988	127	37.20	37.30	37.3
77x16	M	AHQ/ARR	07/05/2003	24/02/2004	293	0.92	4230	4260	177	62.30	62.60	62.5
77x15	M	AHQ/ARR	07/05/2003	24/02/2004	293	1.39	4056	4014	168	54.50	54.70	54.6
76x87	M	VRQ/AHQ	07/05/2003	26/08/2004	477	-	55	67	3	25.00	25.20	25.1
77x17	M	VRQ/ARR	07/05/2003	26/08/2004	477	-	1045	1059	44	38.20	38.20	38.2
77x12	F	VRQ/ARR	01/05/2003	26/08/2004	483	-	818	798	34	31.00	30.50	30.8
77x09	F	AHQ/ARR	01/05/2003	26/08/2004	483	-	191	205	8	16.90	17.30	17.1
76x97	M	VRQ/ARR	03/05/2003	27/10/2004	543	-	864	840	36	5.12	6.20	5.7
77x02	F	VRQ/ARR	03/05/2003	27/10/2004	543	-	240	240	10	2.62	3.10	2.9
76x36	F	ARQ/ARR	05/04/2003	05/10/2005	914	-	125	118	5	4.50	3.85	4.2
76x55	F	ARQ/ARR	05/04/2003	05/10/2005	914	-	80	92	4	0.23	0.20	0.2
76x34	F	ARQ/AHQ	16/04/2003	05/10/2005	903	-	720	741	30	20.00	18.21	19.1
61x62	F	positive	21/04/1998	05/03/2003	1779	-	72	69	3	1.34	1.44	1.4
58x68	M	ARR/ARR	01/04/1997	25/06/2003	2276	-	2	1	0	0.02	0.01	0.0
J2661	F	-	09/04/1997	24/02/2004	2512	-	115	119	5	1.00	1.10	1.1
57x56	M	AHQ/ARR	30/03/1996	25/06/2003	2643	-	0	0	0	0.00	0.00	0.0
54x15	F	negative	18/04/1994	05/03/2003	3243	-	0	0	0	0.30	0.28	0.3
J2035	F	negative	04/04/1992	05/03/2003	3987	-	19	19	1	0.12	0.13	0.1

Appendix B: Immunocytochemistry methods and reagents

B-1 Cutting cryostat sections

1. Place blade in holder.
2. Take piece of tissue trimmed to manageable size.
3. Cover chuck in Tissue-Tek (let it get tacky). Put tissue on chuck and surround with Tissue-Tek.
4. Once Tissue-Tek is dried, place chuck in holder and set cryostat to cut sections at 20 μm.
5. Trim without roll plate until tissue is visible.
6. Alter cryostat to cut about 10 μm. First, cut a couple of sections without roll plate then put roll plate down.
7. Collect sections sequentially.
8. Air dry sections overnight.

B-2 Immunocytochemistry methods

Double immunolabelling for PrP and FDCs in mouse tissues using cryostat sections

1. Prepare wash buffer (TBS) (see below).
2. Defrost slides for 30 minutes at room temperature.
3. Fix slides in acetone for 10 minutes in fume hood.
4. Air dry slides for 15 minutes.
5. Quick wash in TBS for 2 X 5 minutes.
6. Block in normal goat serum at 1/20 for 20 minutes.
7. Tap off serum and apply primary antibody 1B3 at 1/1000 overnight at room temperature. For controls, use normal rabbit serum at 1/1000.
8. Wash 3 X 5 minutes in TBS.
9. Incubate in secondary antibody, goat anti-rabbit alexa 488 (15 μg/ml) for 1 hour at room temperature.
10. Wash 3 X 5 minutes in TBS.
11. Incubate in second block, normal mouse serum at 1/20 for 20 minutes.
12. Tap off serum and apply second primary antibody, FDCM2 biotin at 1/500 for 1 hour. For controls, use normal rat serum at 1/2000.
13. Wash 3 X 5 minutes in TBS.
14. Apply streptavidin-alexa 694 (2.5 μg to 5 μg/ml) for 1 hour.
15. Mount sections in fluorescent mounting media (DAKO).

Immunolabelling for PrP in sheep tissues using cryostat sections

1. Prepare wash buffer (TBS) (see below).
2. Defrost slides for 30 minutes at room temperature.
3. Fix slides in acetone for 10 minutes in fume hood.
4. Air dry slides for 15 minutes.
5. Quick wash in TBS for 3 X 5 minutes.
6. Block in normal rabbit serum at 1/20 for 15 minutes.
7. Tap off serum and apply primary antibody, BG4 at 1/100 for 1 hour at room temperature. For controls, use normal mouse serum at 1/600.
8. Wash 3 X 5 minutes in TBS.
9. Prepare streptavidin biotinylated alkaline phosphatase (SBAP) at 1/100, 30 minutes before use.
10. Add SBAP to each slide for 45 minutes.
11. Wash 3 X 5 minutes in TBS.
12. Prepare substrate solution (see below) for Vector Red.
13. Add Vector Red to each slide for 5 minutes.
14. Immerse in tap water to stop reaction.
15. Counter stain with haematoxylin for 1 minute. Wash in water and then place in Scott's tap water for 1 minute.
16. Dehydrate in alcohol, clear in xylene and mount slides in DPX.

Immunolabelling for FDCs in sheep tissues using cryostat sections

1. Prepare wash buffer (TBS) (see below).
2. Defrost slides for 30 minutes at room temperature.
3. Fix slides in acetone for 10 minutes in fume hood.
4. Air dry slides for 15 minutes.
5. Quick wash in TBS for 3 X 5 minutes.
6. Block in normal rabbit serum at 1/20 for 15 minutes.
7. Tap off serum and apply primary antibody, D62 at 1/100 for 1 hour at room temperature. For controls, use normal mouse serum at 1/600.
8. Wash 3 X 5 minutes in TBS.
9. Prepare streptavidin biotinylated alkaline phosphatase (SBAP) at 1/100, 30 minutes before use.
10. Add SBAP to each slide for 45 minutes.
11. Wash 3 X 5 minutes in TBS.
12. Prepare substrate solution (see below) for Vector Red.
13. Add Vector Red to each slide for 5 minutes.
14. Immerse in tap water to stop reaction.

15. Counter stain with haematoxylin for 1 minute. Wash in water and then place in Scott's tap water for 1 minute.
16. Dehydrate in alcohol, clear in xylene and mount slides in DPX.

Method for immunohistoblotting

1. Cut 10 μm frozen tissue sections and apply to PVDF membrane.
2. Air dry for 18-24 hours (time is approximate and not critical).
3. Store at room temperature in box with silica gel until use.
4. Rehydrate membranes for 1 hour in TBST at room temperature.
5. Split samples into + (proteinase K) PK and –PK.
6. Incubate –PK samples 1 hour at room temperature in 100 mM NaOH.
7. Place +PK samples in PK solution (25, 50 and 100 μg/ml) at 37°C. Place in incubator at 37°C and incubate for 3 hours. Gently shake samples at intervals of 15-20 minutes. Do not use the belly dancer.
8. Wash +PK samples 10 x 2 minutes in distilled or de-ionized water at room temperature.
9. Incubate +PK samples in TBST-PMSF (3mM) 20 minutes at room temperature to stop PK activity.
10. Wash +PK samples 10 x 2 minutes in distilled or de-ionized water at room temperature.
11. Incubate +PK samples in tris HCL pH 7.6/GndSCN for 10 minutes at room temperature.
12. Wash +PK samples and –PK samples 10 x 2 minute washes in TBST.
13. Block membranes in 10% BSA in TBS for 1 hour at room temperature.
14. Add primary antibody, R521 at 1/30,000 (diluted in TBST) and incubate overnight at room temperature.
15. Wash membranes 10 x 2 minutes in TBST.
16. Add goat anti-rabbit alkaline phosphatase-conjugated secondary goat anti-rabbit antibody at 1/3000 (diluted in TBST) for 90 minutes at room temperature.
17. Wash membranes 10 x 2 minutes in TBST.
18. Apply NBT/BCIP substrate for 5-30 minutes at room temperature. Do not shake or use belly dancer during incubation.
19. Wash membranes 2 x 5 minutes in distilled or de-ionized water at room temperature (timings are not critical).
20. Air dry sections overnight on blotting paper or filter paper.
21. Once dry, store sections in plastic holders kept within the laboratory.

B-3 Immunolabelling reagents

Methanol/H_2O_2

232 ml methanol + 8 mls H_2O_2

PBS/BSA wash buffer

Dissolve 5g BSA (bovine serum albumin) in 250 mls stock PBS (10x PBS)

Top up to 2599 ml with distilled water

10x PBS

400 g sodium chloride

10 g potassium chloride

57.5 g disodium hydrogen orthophosphate

10 g potassium dihydrogen orthophosphate

5 litres of deionised water

Scott's tap water

8.75 g sodium hydrogen carbonate

50 g magnesium sulphate

2500 ml tap water

Substrate solution for Vector Red

0.343 g tris HCL pH 8.2

25 ml distilled water

TBS/BSA wash buffer

7.45 g tris HCl pH 7.6

8.76 g NaCl

2 g BSA

Trypsin

5.96 g tyzma buffer (pH 7.6)

0.2 gm trypsin

200ml distilled H_2O

Reagents for histoblots

TBST (1 L)

7.45 g Tris pH 7.6

8.76 g NaCl

1 ml Tween 20

Make up to 1.0 L in distilled or de-ionized water

PK digest buffer (100 ml)

0.148 g Tris pH 7.6

0.58 g NaCl

333 µl of 30% stock Brij 35

Make up to 100 ml in distilled or de-ionized water

Proteinase K (PK) solutions

[final PK]	[µl stock PK]
25 µg/ml	12.5 µl PK
50 µg/ml	25 µl PK
100 µg/ml	50 µl PK

For each PK solution, add the corresponding amount of PK to 10 ml in PK digest buffer and pre-warm to 37°C in a water bath.

100 mM NaOH (100 ml)

NaOH = 0.4 g made up to 100 ml in distilled or de-ionized water

Block buffer (20 ml)

2.0 g BSA made up to 20 ml in TBS

GndSCN (5 ml)

0.149 g Tris pH 7.6 to 100 ml distilled or de-ionized water

Add 1.77 g GndSCN to 5 ml of above

PMSF solution

0.3 M PMSF in propan-1-ol

5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (NBT/BCIP) tablets

1 tablet per 10 ml distilled or de-ionized water

Appendix C: Histoblot analysis

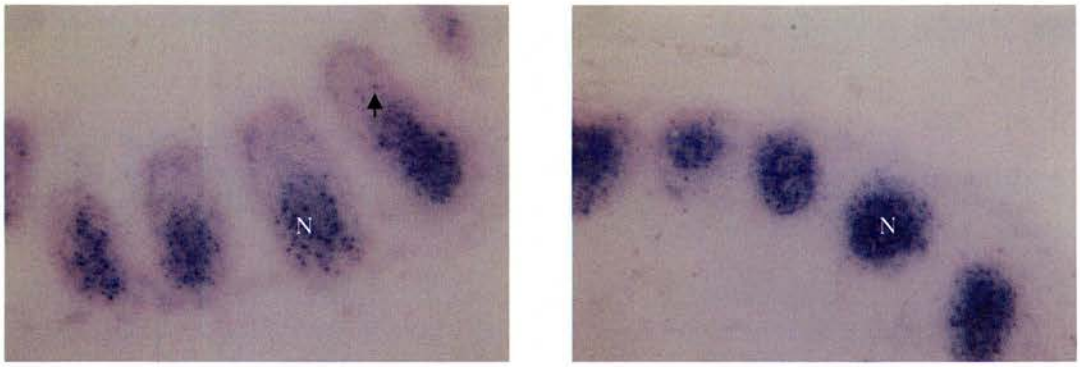

Figure C-1 Higher magnification (X50) **(A)** of the same section of ileal PP tissue in **Figure 5.10D** showing dense accumulation of staining in nodules (N). Arrow indicates pin-point areas of staining that may represent macrophages. Higher magnification **(B)** of same section of mesenteric lymph node **Figure 5.10H** showing several nodules (N) with intense staining.

BMC Infectious Diseases

BioMed Central

Research article

Open Access

Comparative evidence for a link between Peyer's patch development and susceptibility to transmissible spongiform encephalopathies

Suzanne G St Rose*[1], Nora Hunter[2], Louise Matthews[3], James D Foster[2], Margo E Chase-Topping[1], Loeske EB Kruuk[4], Darren J Shaw[5], Susan M Rhind[6], Robert G Will[7] and Mark EJ Woolhouse[1]

Address: [1]Centre for Infectious Diseases, University of Edinburgh, Ashworth Laboratories, King's Buildings, West Mains Road, Edinburgh EH9 3JF, UK, [2]Institute for Animal Health, Neuropathogenesis Unit, West Mains Road, Edinburgh EH9 3JF, UK, [3]Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, UK, [4]Institute of Evolutionary Biology, School of Biological Sciences, University of Edinburgh, Edinburgh EH9 3JT, UK, [5]Division of Veterinary Clinical Studies, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Easter Bush Veterinary Centre, Roslin, Midlothian EH25 9RG, UK, [6]Division of Animal Health and Welfare, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Easter Bush, Roslin, Midlothian EH25 9RG, UK and [7]The National Creutzfeldt-Jakob disease Surveillance Unit, Western General Hospital, Crewe Road, Edinburgh EH4 2XU, UK

Email: Suzanne G St Rose* - s0231156@sms.ed.ac.uk; Nora Hunter - nora.hunter@bbsrc.ac.uk; Louise Matthews - l.matthews@vet.gla.ac.uk; James D Foster - jim.foster@bbsrc.ac.uk; Margo E Chase-Topping - margo.chase@ed.ac.uk; Loeske EB Kruuk - Loeske.Kruuk@ed.ac.uk; Darren J Shaw - Darren.Shaw@ed.ac.uk; Susan M Rhind - Susan.Rhind@ed.ac.uk; Robert G Will - r.g.will@ed.ac.uk; Mark EJ Woolhouse - Mark.Woolhouse@ed.ac.uk

* Corresponding author

Published: 11 January 2006

BMC Infectious Diseases 2006, **6**:5 doi:10.1186/1471-2334-6-5

Received: 14 September 2005
Accepted: 11 January 2006

This article is available from: http://www.biomedcentral.com/1471-2334/6/5

Abstract

Background: Epidemiological analyses indicate that the age distribution of natural cases of transmissible spongiform encephalopathies (TSEs) reflect age-related risk of infection, however, the underlying mechanisms remain poorly understood. Using a comparative approach, we tested the hypothesis that, there is a significant correlation between risk of infection for scrapie, bovine spongiform encephalopathy (BSE) and variant CJD (vCJD), and the development of lymphoid tissue in the gut.

Methods: Using anatomical data and estimates of risk of infection in mathematical models (which included results from previously published studies) for sheep, cattle and humans, we calculated the Spearman's rank correlation coefficient, r_s, between available measures of Peyer's patch (PP) development and the estimated risk of infection for an individual of the corresponding age.

Results: There was a significant correlation between the measures of PP development and the estimated risk of TSE infection; the two age-related distributions peaked in the same age groups. This result was obtained for each of the three host species: for sheep, surface area of ileal PP tissue vs risk of infection, $r_s = 0.913$ ($n = 19$, $P < 0.001$), and lymphoid follicle density vs risk of infection, $r_s = 0.933$ ($n = 19$, $P < 0.001$); for cattle, weight of PP tissue vs risk of infection, $r_s = 0.693$ ($n = 94$, $P < 0.001$); and for humans, number of PPs vs risk of infection, $r_s = 0.384$ ($n = 46$, $P = 0.008$). In addition, when changes in exposure associated with BSE-contaminated meat were accounted for, the two age-related patterns for humans remained concordant: $r_s = 0.360$ ($n = 46$, $P = 0.014$).

Conclusion: Our findings suggest that, for sheep, cattle and humans alike there is an association between PP development (or a correlate of PP development) and susceptibility to natural TSE infection. This association may explain changes in susceptibility with host age, and differences in the age-susceptibility relationship between host species.

Background

The incidence of natural cases of transmissible spongiform encephalopathies (TSEs) or prion diseases is related to age: scrapie incidence in sheep typically peaks between 2 and 3 years of age [1], bovine spongiform encephalopathy (BSE) incidence in cattle peaks at around 5 to 7 years of age [2] and variant Creutzfeldt-Jakob disease (vCJD) incidence in humans peaks at 25 to 30 years [3]. Age-related patterns in incidence will reflect the incubation period of the disease (typically long relative to host life expectancy), the magnitude of the risk of infection and any age dependency in the risk of infection. Analyses of epidemiological data for scrapie [4], BSE [5] and vCJD [6] have suggested that there is significant age dependency in the risk of infection for all these TSEs. Available evidence suggests that these patterns cannot be fully accounted for by changes in exposure, in which case changes in susceptibility must also play a role. However, to date, there has been no indication of why susceptibility might change with age.

Age dependency in the risk of infection by TSEs will reflect any age dependency in exposure to infection and/or in susceptibility to infection for a given level of exposure. Both of these are likely to be linked to the route of transmission. Although other transmission routes may exist (see below), oral exposure appears to be the most important route of transmission for natural TSE infections in sheep, cattle, deer and mink and for vCJD and kuru in humans [1,3,7-9]. There is evidence for the involvement of Peyer's patches (PPs), part of the gut-associated lymphoid tissue (GALT), in orally transmitted TSE infection. Experimental studies in cattle have demonstrated staining for PrP^{Sc} (the abnormal prion protein) in PP follicles in the distal ileum throughout much of the course of the disease following oral exposure to the BSE agent [7]. In sheep, oral infection with scrapie is thought to occur mainly via the ileal PP, followed by replication in GALT [8]. In mule deer fawns, lymphoid follicles of PPs have been shown to accumulate PrP^{Sc} within a few weeks following oral exposure to chronic wasting disease (CWD) [9]. After oral infection of nonhuman primates with BSE-infected material, PrP^{Sc} is initially detected in PPs [10]. In experimental infections, mice deficient in both tumour necrosis factor and lymphotoxin or in lymphocytes, in which PPs are decreased in number, are highly resistant to oral challenge and their intestines are virtually devoid of infectivity at all times post-challenge [11]. These facts collectively suggest a key role for PPs in the infection dynamics of a range of TSEs.

Early presence of PrP^{Sc} in mouse PPs after oral exposure to scrapie [12] has indicated these structures as being the most probable sites for the intestinal uptake of the TSE agent. Various cell types present in this lymphoid tissue have been implicated as important elements in the uptake and propagation of the infectious agent. PrP^{Sc} staining in the follicular dendritic cells of patients with vCJD [13] and of sheep naturally infected with scrapie [14], as well as staining associated with the luminal border of cells in the follicle-associated epithelium (FAE) of sheep suggest uptake of the TSE agent from the intestinal lumen to the underlying lymphoid tissue [8]. Although important functional differences exist between PP in sheep ileum and those in the duodenum and jejunum, the FAE overlying jejunal and ileal PPs has an efficient mechanism for the transcytosis of luminal material [15,16], including prion proteins, to the underlying lymphoid tissue.

The development of GALT is known to be related to age. In young sheep, cattle and humans, ileal PPs are the major component of GALT possessing an extensive bed of follicular dendritic cells and follicle-associated epithelium. The involution of ileal PPs occurs at around puberty in sheep, cattle and humans [17-19]. However, the age-related changes in PP development are not identical across these three species, providing an opportunity for a comparative study. Our hypothesis is that although the relationships between PP development and age and between susceptibility to TSE infection and age differ in sheep, cattle and humans, there should still be a correlation between PP development and susceptibility for each species.

Methods

Anatomical studies

Specimens of ileum were collected from 19 sheep of different ages (0–1 year, 1–2 years and >2 years) from a flock of Cheviot sheep maintained by the Institute of Animal Health Neuropathogenesis Unit (NPU) [20]. The study was limited to animals with no clinical or pathological evidence of intestinal disease. Specimens were obtained from sheep that were either euthanized because of severe arthritis in one or more limbs, died shortly after birth or were culled for flock management reasons. The specimens were opened along their mesenteric borders, and rinsed in cold water. PP tissue and lymphoid follicles were visualised by immersing the intestines in 2% acetic acid for 24 hours, and the follicular content of the patches enhanced by staining with 0.5% methylene blue for 2–5 minutes. PP tissue and lymphoid follicles were easily visualised using this technique.

The terminal ileum (distal 0.6 m of the ileum) was transilluminated on a horizontal X-ray view box and digital images were obtained. Image analysis software (Image-Pro Plus®) was then used to calculate the areas of intestine and of PP tissue. The area of PP tissue was recorded as a percentage of the total area of intestinal tissue.

To determine the number of lymphoid follicles, the stained intestine was placed between two glass slides, the upper of which was etched in square centimetres. Individual lymphoid follicles appeared as bright blue spots against a faintly blue background when viewed on the X-ray box. The number of lymphoid follicles in 6 different sections along the length of the terminal ileum was counted by naked eye, starting at 5 cm from its caudal end and selecting 4 cm^2 sections at every 10 cm thereon, proximally. Results were recorded as the average number of lymphoid follicles per cm^2 of ileum.

Our results are described in terms of area of PP tissue and lymphoid follicle density in the sheep ileum; analyses indicate that these two measures are closely correlated (r_s = 0.958, n = 19, $P < 0.001$). PP data for cattle and humans were obtained from earlier studies [18,19]. The studies used different measures to quantify PP tissue from those we obtained here for sheep. The cattle data [18] refer to weight of PP tissue in the small intestine of 94 German beef cattle. The human data [19] refer to number of PPs in the normal small intestine of 46 individuals between 15 and 96 years of age. The study was limited to necropsies performed within a few hours of death, and to patients with no clinical history or pathological evidence of gastrointestinal tract disease. A second, smaller study of human PPs indicates that, in humans, number of PPs and area of PP tissue in the distal ileum were correlated across age classes ($r = 0.415$, $n = 55$, $P < 0.01$) [21]. As far as we are aware, there are no other quantitative data on PP development with respect to age available for these species but, where direct comparisons are possible, it appears that the different measures reflect the same underlying relationship with age.

Scrapie incidence data

The NPU Cheviot flock, a closed flock maintained explicitly as a source of natural scrapie infections, has been comprehensively documented and demographic information and epidemiological data on all sheep are available [20]. In this study, analyses were based on data obtained from an outbreak of scrapie, which spanned the years 1985 to 1994 affecting cohorts born between 1983 and 1992. This represents a total of 1,473 sheep of which 34 developed clinical scrapie. In this flock, scrapie occurs in two PrP genotypes, VRQ/VRQ and VRQ/ARQ [20]. (There is no evidence that PrP genotype influences PP development). Further details of the outbreak are given elsewhere [20].

Age-susceptibility functions

The method for calculating the age susceptibility function for sheep follows that of Boëlle et al. [6] used to derive the age risk function for vCJD. The occurrence of cases in genotype G sheep is modelled by a Poisson process in the (age, time) plane with intensity $\pi_G(a,t)$ given by:

$$\pi_G(a,t) = \beta_G r_G S(a) \int_0^a \exp\left[-\int_0^{a'} \lambda(u, t-a+u)du \right] \lambda(a', t-a+a') h_G(a-a')da'$$

where β_G is the birth rate and r_G is the relative susceptibility of genotype G individuals, $S(a)$ is the probability of survival (in the absence of scrapie) until age a, h_G is the probability density function for the incubation period for genotype G individuals, and $\lambda(a,t)$ is the per capita rate of infection for individuals of age a at time t. The expression sums the contribution to the incidence of infection at age a and time t from animals infected when at age a', taking into account the fact that the number of animals available at age a' to become infected is reduced by those already infected at age u. The low incidence of scrapie in this flock [22] permits modelling of the age and timing of cases as a Poisson process because the course of the outbreak does not significantly impact on the demography of the susceptible sheep.

The survivorship function $S(a)$ is a Weibull function with mean age of death of 2.99 years [23]. The incubation period distribution is a gamma distribution with a mean of 1.9 years [23]. The birth rate β_G is selected to give the average numbers of sheep of different genotypes born per year. The per capita rate of infection, $\lambda(a,t)$ has two parts: a time dependent component $g(t)$ which is assumed here to be proportional to an exponential function fitted to the incidence of infection; and an age-dependent component f(a) which represents the relative susceptibilities of different age classes:

$$f(a) = \begin{cases} f_1 & \text{for} \quad 0 \le a < 1 \\ f_2 & \text{for} \quad 1 \le a < 2 \\ f_3 & \text{for} \quad 2 \le a < 9 \end{cases}$$

where the maximum value taken by f_1 f_2 or f_3 is equal to 1. Standard theory on point processes [24], gives the log-likelihood of the observed age-of-case data to be:

$$\sum_i^{N_{cases}} \log(\pi_{G_i}(a_i, t_i)) - \sum_G \int\int \pi_G(a,t) da dt$$

The subscript i denotes actual case data; deaths are known to occur at age a_i and a time t_i after the start of the outbreak. Maximum likelihood methods were used to estimate the constant of proportionality, which determines the magnitude of the per capita rate of infection and the age-dependent susceptibility function as defined by f_1, f_2 and f_3. We did this for (i) the 34 cases over the 10 year period assuming no differences between genotypes, and (ii) for the 28 genotyped cases allowing the 8 VRQ/ARQ cases to have either a lower susceptibility to infection or (iii) a longer incubation period than the 20 VRQ/VRQ

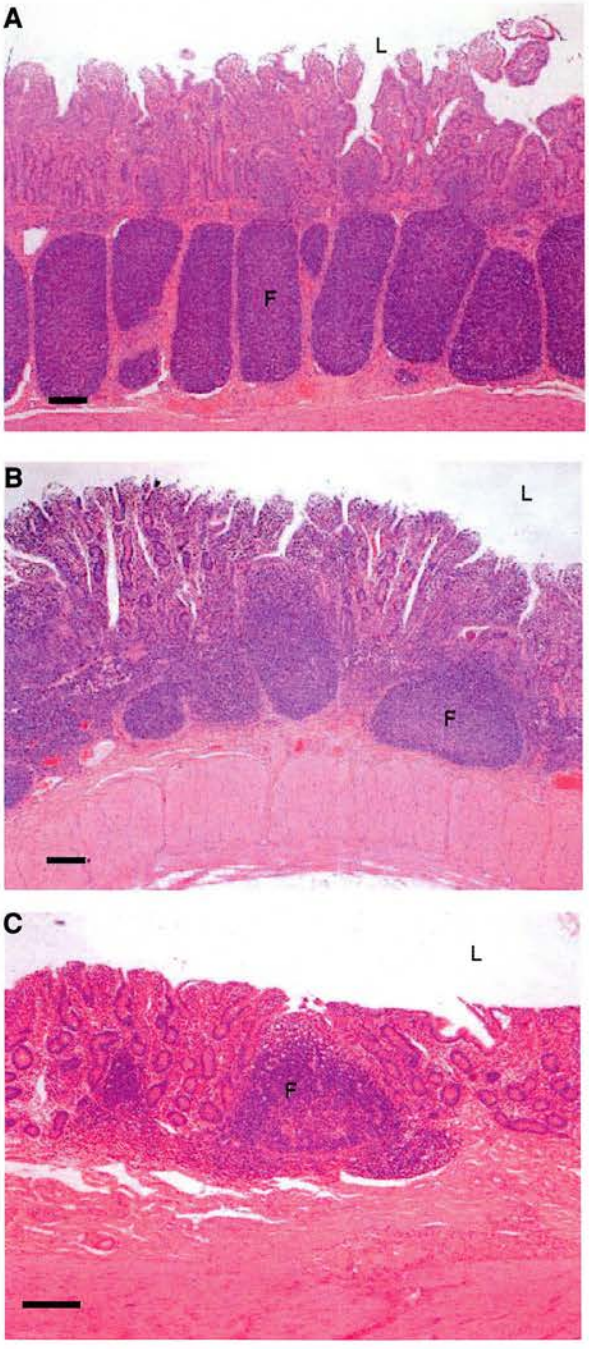

Figure 1
Comparison of Peyer's patch lymphoid follicles in the ileum of NPU Cheviot sheep at (A) 4 months, (B) 15 months, and (C) 6 years, using haematoxylin and eosin staining. F, lymphoid follicles undergo involution and are fewer in number with increasing age; L, intestinal lumen. Bar = 200 μm.

cases. We found that models (ii) and (iii) produced a significant improvement in fit at the 95% level over model (i), but that the shape of the age-dependent susceptibility function was robust to the choice of model. Results are shown for model (ii).

For cattle, estimates of risk of BSE infection were made from n = 158,550 BSE cases in British cattle and were calculated from the cumulative distribution function, defined by Ferguson et al. [5], corresponding to the age-exposure/susceptibility curve (fitted using maximum likelihood methods).

For humans, estimates of risk of vCJD infection were obtained from a previous study that comprised n = 129 vCJD cases in British people, and were fitted using maximum likelihood methods by Boëlle et al. [6].

Concordance between susceptibility data and anatomical data

For each combination of anatomical data and risk of infection estimates we calculated the Spearman's rank correlation coefficient, r_s, between the value of the available measure of PP development (area, weight or number) and the risk of infection for an individual of the corresponding age. Sample sizes were $n = 19$, $n = 94$ and $n = 46$ for sheep, cattle and humans, respectively. Correlation coefficients were calculated using S-PLUS 2000 for Windows.

Results

For sheep, cattle and humans alike, a strong correlation was found between risk of TSE infection and the development of lymphoid tissue in the gut which can explain both the relationships between age and disease incidence within species and differences in this relationship between species.

For sheep there is a marked fall in both the surface area of ileal PP tissue and lymphoid follicle density between approximately 12 and 24 months old, and both measures remain very low throughout adulthood (Figure 1 and 2(A)). Analysis of data on the incidence of natural scrapie over a 10 year period in the sheep flock providing the anatomical data indicates that the risk of infection is highest in the first year of life and is lowest in sheep >2 years old (Figure 2(A)). The two distributions peak in the same age class and are highly concordant (see Methods): surface area of ileal PP tissue vs risk of infection, $r_s = 0.913$ ($n = 19$, $P < 0.001$); lymphoid follicle density vs risk of infection, $r_s = 0.933$ ($n = 19$, $P < 0.001$).

For cattle, previous work [18] has shown that the weight of PP tissue in the small intestine increases in the first year of life, peaks at 12–18 months old, declines thereafter, and is low throughout adulthood (Figure 2(B)). Available

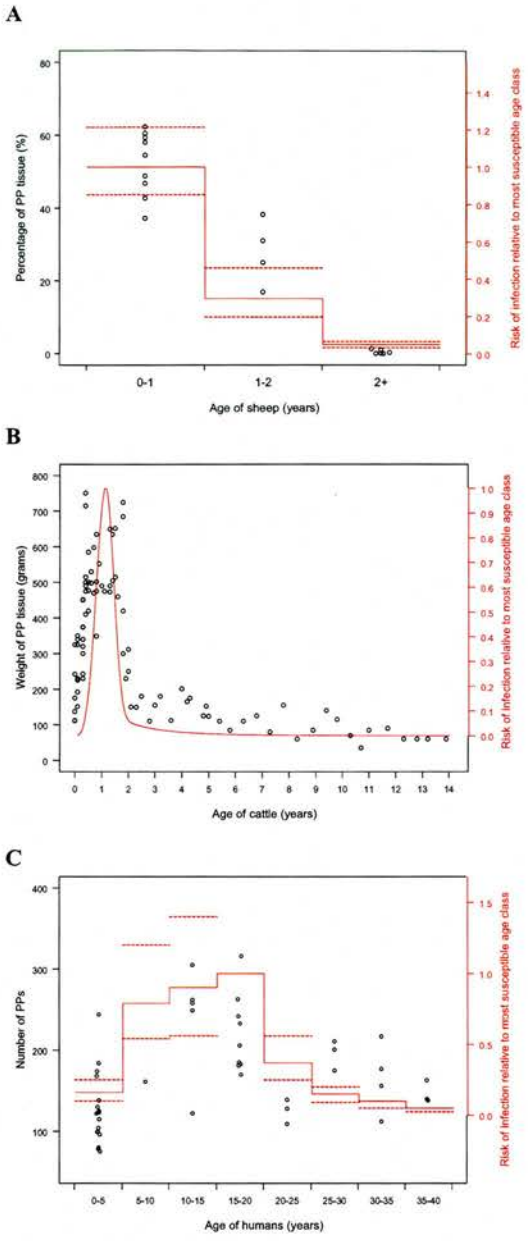

Figure 2
Comparison of age-related changes in Peyer's patch (PP) development and estimated risk of TSE infection relative to the most susceptible age class, for sheep, cattle and humans. (A) PP areas for n = 19 Cheviot sheep of mixed genotypes in 3 age classes (left hand axis, open circles), compared with estimates of risk of scrapie infection relative to the most susceptible age class (solid line) (± 50 percentiles-dashed lines) from field data on n = 34 cases in mixed PrP genotype (VRQ/VRQ and VRQ/ARQ) Cheviot sheep (see Methods) in the same age classes (right hand axis). (B) PP tissue weight against age for n = 94 cattle (open points, data from ref. 18), compared with estimates of risk of BSE infection relative to the most susceptible age class (solid line) as a function of age made from n = 158,550 BSE cases in British cattle [5] (C) Numbers of PPs in the small intestine in 8 age classes of humans (open circles, data taken from ref. 19), compared with estimates of risk of vCJD infection relative to the most susceptible age class (solid line, ± 50 percentiles-dashed lines) from n = 129 vCJD cases in British people for the same age classes (redrawn from ref. 6).

estimates of age-related risk of infection of the British cattle population with BSE up to 1996 (given a mean incubation period of 5 years; published estimates range from 4.5 to 5.5 years [25]) indicate that the risk is initially low, peaks at about 12 months, and declines rapidly thereafter (Figure 2(B)). Again, the two distributions peak at similar ages and are concordant: $r_s = 0.693$ ($n = 94$, $P < 0.001$).

For humans, previous work [19] has shown that the number of PPs in the small intestine increases during childhood, peaks at 10–15 years old, and declines thereafter, although the PPs persist throughout adulthood (Figure 2(C)). Recent estimates of age-related risk of infection of the British human population to vCJD [6] indicate that the risk is initially low, peaks between 5 and 20 years, and declines thereafter (Figure 2(C)). Here too, the two age-related patterns are concordant: $r_s = 0.38$ ($n = 46$, $P = 0.008$). The same study [6] provides estimates of age-related susceptibility having allowed for changes in putative exposure associated with consumption of bovine carcass meat (see below). This is also concordant with the number of PPs: $r_s = 0.360$ ($n = 46$, $P = 0.014$). Importantly, these correlations occur despite the markedly different patterns of age-related development of GALT in humans as compared with sheep and cattle.

Discussion

Our results show that, whilst both age-related changes in the development of PP tissue and estimated risks of TSE infection differ between sheep, cattle and humans, in each case the two are associated. However, these results do not distinguish effects of age-related changes in exposure to TSE infection from age-related changes in susceptibility. To make this distinction we need to consider how oral exposure to TSE infection might change with age for each species.

For BSE in cattle, epidemiological studies have implicated meat and bone meal (MBM) containing recycled infected cattle tissues [26]. MBM used to be incorporated as a protein source in concentrated feedstuffs and fed to both calves and adult cattle. However, there is no clear correlation with the estimated age-infection function (Figure 2(B)): almost all calves were exposed to MBM by 6 weeks of age; exposure then fluctuated up to 24 months old but, especially for dairy cows, rose again in adulthood [27,28]. This route of BSE transmission is thought now to have been eliminated by feed production regulations introduced in 1988 and 1996.

For vCJD in humans, the most likely vehicle for exposure is food products containing BSE-contaminated cattle tissues [29]. Humans consume solid foods from 4–6 months of age with average consumption of bovine carcass meat peaking during childhood and tending to fall thereafter (see Figure 3 in [6]). This route of transmission is thought now to have been eliminated by food production regulations introduced in the UK in 1996. Here, putative exposure is more closely aligned with PP development [6] but, as reported above, when age-related exposure is taken into account, there remains an association between PP development and estimated susceptibility.

For scrapie in sheep, the vehicle(s) of oral exposure are less well understood, but are likely to include grazing on pasture contaminated with scrapie, possibly by infected foetal membranes [30]. Lambs typically begin to graze at 6–14 weeks and continue to do so throughout their lives. Exposure by this route would not be correlated with the estimated age-infection function (Figure 2(A)).

The importance of other transmission routes is less clear. Transmission from mother to offspring in utero or via breast milk (self-evidently age-dependent) is thought to play a minor role, if any: currently available estimates of the fraction of cases due to maternal transmission are 0–8% for scrapie in sheep [23], 0–14% for BSE in cattle [31], and 0% for vCJD in humans (Will et al., unpublished data). Other suggested routes include skin scarification (as demonstrated experimentally in mice [32]), food-borne infection via oral lesions [33], for scrapie possibly even mechanical transmission involving arthropods [34], and for vCJD, iatrogenic transmission [3]. However, there is no evidence that exposure via any of these routes varies with age in a manner corresponding to the estimated risk of infection functions (Figure 2)

The measures of PP development (area, weight or number) used in this study are crude indicators of lymphoid tissue development; alternative measures in PP development may be at least as appropriate (for example, in sheep, counts of functionally mature FDCs). Moreover, this analysis assumes that both the anatomical data and the age-susceptibility estimates available are representative of each host species in general and not just the specific populations examined. Similarly, it is assumed that the associations studied have not been distorted by other factors (e.g. history of exposure to gut pathogens) which might influence PP development and/or susceptibility to TSEs.

Given these caveats, it is nonetheless striking that an association between PP development and susceptibility to TSEs is seen not just in one host species but in three host species with different relationships between these variables and age. This kind of comparative study is especially useful in cases such as this where experimental manipulations (e.g. of PP development) are not feasible.

Conclusion

Taken together, the epidemiological, anatomical and pathological evidence are consistent with the hypothesis that PP development or a close correlate of PP development is a major determinant of the observed age distribution of natural cases of TSEs in sheep, cattle and humans. This implies that the age groups most at risk of TSE infection (given that the individuals are exposed and have a susceptible PrP genotype) are indicated by the development of Peyer's patches in the gut.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

SGS participated in all aspects of the study, anatomical studies of sheep and preparation of the manuscript. NH collected the sheep data, provided advice on database information and participated in the interpretation of findings. LM participated in the data analysis and interpretation of findings. JDF participated in the collection of data and interpretation of findings. MECT participated in the analysis, interpretation and presentation of findings. LEBK participated in the data analysis and interpretation of findings. DJS participated in the analysis, interpretation and presentation of findings. SMR participated in the acquisition and interpretation of gross and anatomical data. RGW participated in the interpretation of findings and preparation of the manuscript. MEJW participated in the interpretation of findings and preparation of the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We thank Dawn Drummond, David Parnham and Neil MacIntyre for technical assistance. Many thanks to Calum McKenzie for assistance on the farm and sheep care. This work was funded by the BBSRC and Wellcome Trust.

References

1. Detweiler LA, Baylis M: **The epidemiology of scrapie.** *Rev Sci Tech OIE* 2003, **22:**121-143.
2. Defra: . [http://www.defra.gov.uk/animalh/bse/statistics/incidence.html].
3. Will RG: **Acquired prion disease: iatrogenic CJD, variant CJD, kuru.** *Brit Med Bull* 2003, **66:**255-265.
4. Matthews L, Coen PG, Foster JD, Hunter N, Woolhouse ME: **Population dynamics of a scrapie outbreak.** *Archives of Virology* 2001, **146:**1173-1186.
5. Ferguson NM, Donnelly CA, Woolhouse ME, Anderson RM: **The epidemiology of BSE in cattle herds in Great Britain. II. Model construction and analysis of transmission dynamics.** *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences* 1997, **352:**803-838.
6. Boëlle PY, Cesbron JY, Valleron AJ: **Epidemiological evidence of higher susceptibility to vCJD in the young.** *BMC Infect Dis* 2004, **4:**26-32.
7. Terry LA, Marsh S, Ryder SJ, Hawkins SA, Wells GA, Spencer YI: **Detection of disease-specific PrP in the distal ileum of cattle exposed orally to the agent of bovine spongiform encephalopathy.** *Vet Rec* 2003, **152:**387-392.
8. Heggebo R, Press CM, Gunnes G, Lie KI, Tranulis MA, Ulvund M, Groschup MH, Landsverk T: **Distribution of prion protein in the ileal Peyer's patch of scrapie-free lambs and lambs naturally and experimentally exposed to the scrapie agent.** *J Gen Virol* 2000, **81:**2327-2337.
9. Sigurdson CJ, Williams ES, Miller MW, Spraker TR, O'Rourke KI, Hoover EA: **Oral transmission and early lymphoid tropism of chronic wasting disease PrPres in mule deer fawns (Odocoileus hemionus).** *J Gen Virol* 1999, **80:**2757-2764.
10. Bons N, Mestre-Frances N, Belli P, Cathala F, Gajdusek DC, Brown P: **Natural and experimental oral infection of nonhuman primates by bovine spongiform encephalopathy agents.** *Proc Natl Acad Sci U S A* 1999, **96:**4046-4051.
11. Prinz M, Huber G, Macpherson AJ, Heppner FL, Glatzel M, Eugster HP, Wagner N, Aguzzi A: **Oral prion infection requires normal numbers of Peyer's patches but not of enteric lymphocytes.** *Am J Pathol* 2003, **162:**1103-1011.
12. Beekes M, McBride PA: **Early accumulation of pathological PrP in the enteric nervous system and gut-associated lymphoid tissue of hamsters orally infected with scrapie.** *Neuroscience Letters* 2000, **278:**181-184.
13. Hill AF, Butterworth RJ, Joiner S, Jackson G, Rossor MN, Thomas DJ, Frosh A, Tolley N, Bell JE, Spencer M, King A, Al-Sarraj S, Ironside JW, Lantos PL, Collinge J: **Investigation of variant Creutzfeldt-Jakob disease and other human prion diseases with tonsil biopsy samples.** *Lancet* 1999, **353:**183-189.
14. van Keulen LJ, Schreuder BE, Meloen RH, Mooij-Harkes G, Vromans ME, Langeveld JP: **Immunohistochemical detection of prion protein in lymphoid tissues of sheep with natural scrapie.** *J Clin Microbiol* 1996, **34:**1228-1231.
15. Griebel PJ, Hein WR: **Expanding the role of Peyer's patches in B-cell ontogeny.** *Immunol Today* 1996, **17:**30-39.
16. Lie KI, Aleksandersen M, Landsverk T: **Lymphoid follicles of different phenotype appear in ileum during involution of the sheep ileal Peyer's patch.** *Dev Comp Immunol* 2005, **29:**539-553.
17. Reynolds JD, Morris B: **The evolution and involution of Peyer's patches in fetal and postnatal sheep.** *Eur J Immunol* 1983, **13:**627-635.
18. Carlens O: **Studien über das lymphatische gewebe des darmkanals bei einigen haustieren, mit besonderer berücksichtingung der embryonalen entwicklung, der mengenverhaltnisse und der altersinvolution dieses gewebs im dünndarm des rindes.** *Z Anat Entwicklungs* 1928, **86:**393-493.
19. Cornes JS: **Number, size, and distribution of Peyer's patches in human small intestine.** *Gut* 1965, **6:**225-233.
20. Hunter N, Foster JD, Goldmann W, Stear MJ, Hope J, Bostock C: **Natural scrapie in a closed flock of Cheviot sheep occurs only in specific PrP genotypes.** *Arch Virol* 1996, **141:**809-824.
21. van Kruiningen HJ, West AB, Freda BJ, Holmes KA: **Distribution of Peyer's patches in the distal ileum.** *Inflamm Bowel Dis* 2002, **8:**180-185.
22. Cox DR, Isham V: **Point Processes.** London, Chapman and Hall; 1980.
23. Arnold ME, Wilesmith JW: **Estimation of the age-dependent risk of infection to BSE of dairy cattle in Great Britain.** *Prev Vet Med* 2004, **66:**35-47.
24. Wilesmith JW, Wells GA, Cranwell MP, Ryan JB: **Bovine spongiform encephalopathy: epidemiological studies.** *Vet Rec* 1988, **123:**638-644.
25. Anderson RM, Donnelly CA, Ferguson NM, Woolhouse ME, Watt CJ, Udy HJ, MaWhinney S, Dunstan SP, Southwood TR, Wilesmith JW, Ryan JB, Hoinville LJ, Hillerton JE, Austin AR, Wells GA: **Transmission dynamics and epidemiology of BSE in British cattle.** *Nature* 1996, **382:**779-788.
26. Wilesmith JW, Wells GAH, Ryan JBN, Gavier-Widen D, Simmons MM: **A cohort study to examine maternally-associated risk factors for BSE.** *Vet Rec* 1997, **141:**239-243.
27. Cooper JD, Bird SM: **UK dietary exposure to BSE in beef mechanically recovered meat: by birth cohort and gender.** *J Cancer Epidemiol and Prev* 2002, **7:**59-70.
28. Pattison IH, Hoare MN, Jebbett JN, Watson WA: **Spread of scrapie to sheep and goats by oral dosing with foetal membranes from scrapie-affected sheep.** *Vet Rec* 1972, **90:**465-468.
29. Gore SM, Gilks WR, Wilesmith JW: **BSE maternal cohort study: explanatory analysis.** *Appl Stat* 1997, **46:**305-320.

30. Taylor DM, McConnell I, Fraser H: **Scrapie infection can be established readily through skin scarification in immunocompetent but not immunodeficient mice.** *J Gen Virol* 1996, **77:**1595-1599.
31. Bartz JC, Kincaid AE, Bessen RA: **Rapid prion neuroinvasion following tongue infection.** *J Virol* 2003, **77:**583-591.
32. Lupi O: **Could ectoparasites act as vectors for prion diseases?** *Int J Dermatol* 2003, **42:**425-429.

Pre-publication history

The pre-publication history for this paper can be accessed here:

http://www.biomedcentral.com/1471-2334/6/5/prepub